Title: METHODS FOR DIAGNOSIS AND TREATMENT OF NEURODEGENERATIVE DISEASES OR DISORDERS

Abstract: The present invention provides methods that are useful for the diagnosis of neurodegenerative disease or disorder and for the screening of compounds or therapeutic agents for treating a neurodegenerative disease or disorder. The methods pertain in part to the correlation of a neurodegenerative disease or disorder with abnormal or altered endoplasmic reticulum-mitochondrial-associated membranes (ER-MAM) integrity.
METHODS FOR DIAGNOSIS AND TREATMENT OF NEURODEGENERATIVE DISEASES OR DISORDERS

[0001] This patent disclosure contains material that is subject to copyright protection. The copyright owner has no objection to the facsimile reproduction by anyone of the patent document or the patent disclosure as it appears in the U.S. Patent and Trademark Office patent file or records, but otherwise reserves any and all copyright rights.

[0002] This invention was made with government support under NS39854 and HD32062 awarded by the National Institutes of Health. The government has certain rights in the invention.


[0004] All patents, patent applications and publications cited herein are hereby incorporated by reference in their entirety. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described herein.

BACKGROUND OF THE INVENTION

[0005] Neurodegenerative diseases are a major public health concern. The increasing number of patients with neurodegenerative diseases imposes a major financial burden on health systems around the world.

39:168-177), can be predisposing genetic factors. At least three genes have been identified in the familial form (FAD): amyloid precursor protein (APP), presenilin-1 (PS1), and presenilin-2 (PS2).

[0007] More than half of the patients with dementia have Alzheimer's disease (AD). The prevalence for AD between the age 60-69 years is 0.3%, 3.2% between that age 70-79 years, and 10.8% between 80-89 years of age (Rocca, Hofman et al. 1991). Survival time after the onset of AD is in the range of 5 to 12 years (Friedland, 1993).

[0008] Although various diagnostic tests exist to detect AD (see U.S. Pat. Nos. 5,508,167, 6,451,547, 6,495,335 and 5,492,812), a major hurdle in developing anti-AD drugs has the lack of a defined causative event in the genesis of the disease. Thus there remains a need for improved methods diagnosis of AD and for methods to identify compounds suitable for the treatment, prevention or inhibition of AD.

SUMMARY OF THE INVENTION

[0009] The present invention provides methods that are useful for the diagnosis of Alzheimer's disease (AD) and for the screening of compounds or therapeutic agents for treating AD. The methods pertain in part to the correlation of AD with abnormal or altered endoplasmic reticulum-mitochondrial-associated membranes (ER-MAM) integrity.

[0010] In one aspect, the invention provides a method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising: (a) obtaining a test biological sample from a subject, (b) measuring the rate of conversion of phosphatidyserme to phosphatidylethanolamme in the test biological sample of step (a), (c) comparing the rate of conversion of phosphatidyserme to phosphatidylethanolamme measured in step (b) to the rate of conversion of phosphatidyserme to phosphatidylethanolamme in a control biological sample, wherein a greater rate of conversion of phosphatidyserme to phosphatidylethanolamme measured in the test biological sample of step (b) relative to the rate of conversion of phosphatidyserme to phosphatidylethanolamme measured in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

[0011] In one aspect, the invention provides a method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising: (a) obtaining a test biological sample from a subject, (b) measuring the amount of localization of ER-MAM
associated proteins to ER-MAM in the test biological sample of step (a), (c) comparing the amount of localization of ER-MAM associated proteins to ER-MAM measured in step (b) to the amount of localization of ER-MAM associated proteins to ER-MAM in a control biological sample, wherein an altered amount of localization of ER-MAM associated proteins to ER-MAM measured in the test biological sample of step (b) relative to the amount of localization of ER-MAM associated proteins to ER-MAM in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

[0012] In one aspect, the invention provides a method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising: (a) obtaining a test biological sample from a subject, (b) measuring the amount of ER-MAM in the test biological sample of step (a), (c) comparing the amount of ER-MAM measured in step (b) to the amount of ER-MAM in a control biological sample, wherein an altered amount of ER-MAM measured in the test biological sample of step (b) relative to the amount of ER-MAM in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

[0013] In one aspect, the invention provides a method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising: (a) obtaining a test biological sample from a subject, (b) measuring the activity of one or more non-presenilin ER-MAM-associated proteins in the test biological sample of step (a), (c) comparing the activity of one or more non-presenilin ER-MAM-associated proteins measured in step (b) to the activity of one or more non-presenilin ER-MAM-associated proteins in a control biological sample, wherein an altered activity of the one or more non-presenilin ER-MAM-associated proteins measured in the test biological sample of step (b) relative to the activity of the one or more non-presenilin ER-MAM-associated proteins in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

[0014] In one aspect, the invention provides a method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising: (a) obtaining a test biological sample from a subject, (b) measuring the amount of functional presenilin in ER-MAM in the test biological sample of step (a), (c) comparing the amount of functional presenilin in ER-MAM measured in step (b) to the amount of functional presenilin in ER-MAM in a control biological sample, wherein a lesser amount of functional presenilin in ER-MAM measured in the test biological sample of step (b) relative to the amount of functional
presenilin in ER-MAM in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

[0015] In one aspect, the invention provides a method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising: (a) obtaining a test biological sample from a subject, (b) measuring the amount of association between ER-MAM associated proteins in the test biological sample of step (a), (c) comparing the amount of association between ER-MAM associated proteins measured in step (b) to the amount of association between ER-MAM associated proteins in a control biological sample, wherein an altered amount of association between ER-MAM associated proteins measured in the test biological sample of step (b) relative to the amount of association between ER-MAM associated proteins in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

[0016] In one aspect, the invention provides a method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising: (a) obtaining a test biological sample from a subject, (b) measuring the sensitivity to cinnamycin induced cell death in the test biological sample of step (a), (c) comparing the sensitivity to cinnamycin induced cell death measured in step (b) to the sensitivity to cinnamycin induced cell death in a control biological sample, wherein a greater sensitivity to cinnamycin induced cell death measured in the test biological sample of step (b) relative to the sensitivity to cinnamycin induced cell death in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

[0017] In one aspect, the invention provides a method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising: (a) obtaining a test biological sample from a subject, (b) measuring the amount of communication between ER and mitochondria in the test biological sample of step (a), (c) comparing the amount of communication between ER and mitochondria measured in step (b) to the amount of communication between ER and mitochondria in a control biological sample, wherein a greater amount of communication between ER and mitochondria measured in the test biological sample of step (b) relative to the amount of communication between ER and mitochondria in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.
In one aspect, the invention provides a method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising: (a) obtaining a test biological sample from a subject, (b) measuring the sensitivity to apoptogen induced cell death in the test biological sample of step (a), (c) comparing the sensitivity to apoptogen induced cell death measured in step (b) to the sensitivity to apoptogen induced cell death in a control biological sample, wherein a greater amount of apoptogen to cinnamycin induced cell death measured in the test biological sample of step (b) relative to the sensitivity to apoptogen induced cell death in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

In one aspect, the invention provides a method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising: (a) obtaining a test biological sample from a subject, (b) measuring the amount of ACATI activity in the test biological sample of step (a), (c) comparing the amount of ACATI activity measured in step (b) to the amount of ACATI activity in a control biological sample, wherein a greater amount of ACATI activity measured in the test biological sample of step (b) relative to the amount of ACATI activity in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

In one aspect, the invention provides a method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising: (a) obtaining a test biological sample from a subject, (b) measuring the amount of cholesterol ester synthesis in the test biological sample of step (a), (c) comparing the amount of cholesterol ester synthesis measured in step (b) to the amount of cholesterol ester synthesis in a control biological sample, wherein a greater amount of cholesterol ester synthesis measured in the test biological sample of step (b) relative to the amount of cholesterol ester synthesis in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

In one aspect, the invention provides a method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising: (a) obtaining a test biological sample from a subject, (b) measuring the amount of lipid droplet formation in the test biological sample of step (a), (c) comparing the amount of lipid droplet formation measured in step (b) to the amount of lipid droplet formation in a control biological sample, wherein a greater amount of lipid droplet formation measured in the test biological sample of
step (b) relative to the amount of lipid droplet formation in the control biological sample indicates that the subject is predisposed to having Alzheimer’s disease.

[0022] In one aspect, the invention provides a method for determining whether a compound ameliorates Alzheimer’s disease in a subject, the method comprising determining whether a test compound is capable of (a) decreasing the amount of phosphatidylserine conversion to phosphatidylethanolamine in cells contacted with the test compound as compared to cells not contacted with the test compound; (b) increasing amount of functional presenilin in ER-MAM in cells contacted with the test compound as compared to cells not contacted with the test compound; (c) decreasing sensitivity to cinnamycin induced cell death in cells contacted with the test compound as compared to cells not contacted with the test compound; (d) decreasing communication between ER and mitochondria in cells contacted with the test compound as compared to cells not contacted with the test compound; (e) decreasing cell death in response to an apoptogen in cells contacted with the test compound as compared to cells not contacted with the test compound; (f) decreasing ACAT1 activity in cells contacted with a test compound as compared to cells not contacted with the test compound; (g) decreasing cholesterol ester synthesis in cells contacted with the test compound as compared to cells not contacted with the test compound; (h) decreasing lipid droplet formation in cells contacted with the test compound as compared to cells not contacted with the test compound, or any combination thereof.

BRIEF DESCRIPTION OF THE FIGURES

[0023] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0024] Fig 1. PS1 fibroblasts are smaller than controls. Both photos at 40x. Red, mitochondria; green, microtubules.

[0025] Fig 2. Control mitochondria are elongated; PS1 mitochondria are more punctate. 100x magnification.

[0026] Fig 3 Western blot analysis of subcellular fractions of mouse liver and brain. Thirty mg of total protein were loaded in each lane and probed with the indicated antibodies.
Fig. 4. Immunohistochemistry to detect PSI in cells. Cells were stained with MT Red (red) and with anti-PSI (green); the merged photo is at bottom (yellow if MT Red and PSI are co-localized). Fig. 4A-B. Comparison of various fixation techniques. Fig 4A. When cells were treated using "standard" techniques (fixation with PF and permeabilization with TX-100), there was poor co-localization of the two signals (the orange staining in the merge panel is the non-specific overlap of the MT Red stain with the diffuse anti-PSI stain). 40x. Fig 4B: However, if TX-100 was replaced with MeOH, whether in the absence or presence of PF, there was excellent colocalization with a subset of mitochondria that are predominantly perinuclear. Asterisks mark mitochondria that are cortical and do not co-localize with PSI. Note that PSI does not stain mitochondria exclusively, as some non-mitochondrial staining is still observed. 40x. Fig 4C-E: Localization of PEMT and PSI in human fibroblasts. MeOH fixation. As in Fig. 4B, both PSI (C) and PEMT (D) co-localized with MT Red, mainly in regions proximal to the nucleus (yellow arrowheads), with a lower degree of co-localization in more distal mitochondria (red arrowheads). 63x. Fig 4E. When stained simultaneously for PEMT (red) and PSI (green), both proteins showed a high degree of co-localization, implying that PSI, like PEMT, is in the MAM. 100x.

Fig 5. Proportion of ER, MAM, and mitochondria in control and FAD fibroblasts. Asterisks denote significance of avg±SD.

Fig 6. Mitochondrial morphology in FADPSI fibroblasts. Fig 6A: Example of staining of control and FADPSI (mutation indicated) fibroblasts with MTred (red) and anti-tubulin (green) (63x). Fig 6B: Mitochondria in control cells have a reticulated network, whereas those in FADPSI (A246E) cells are more punctate (100x). Fig 6C: Example of quantitation of the number of mitochondria located outside the circular region; n, # of cells examined; asterisks denote significance of avg±SEM (p<.05).

Fig 7. Mitochondrial morphology in COS7 cells expressing stably-transfected wild type (WT) or mutated (A246E) PSI stained with MTred (red) and decorated with anti-tubulin (green). Fig 7A: Transfection with empty vector. Fig 7B: Transfection with wild-type PSI. Fig. 7C. Transfection with mutated (A246E) PSI.

Fig 8. Mitochondrial morphology in FADPSI fibroblasts in PSI-knockdown mouse embryonic fibroblasts. Fig 8A: Example of staining of control and FADPSI (A246E) fibroblasts with MTred (red) and anti-tubulin (green) (63x). Fig 8B: Example of quantitation of the number of mitochondria located in the cell periphery (see Methods). Three
replicate experiments were performed; n, number of cells examined; error bars denote standard error of the mean (SEM); asterisks denote significant difference vs. control (p<0.05). Fig. 8C Mitochondria in control cells are a reticulated network, whereas those in FAD<sup>PS1</sup> (A246E) cells are more punctate (100x). Fig 8D: Relative proportion of protein in ER, ER-MAM, and mitochondrial fractions in control and FAD<sup>PS1</sup> (A246E) human fibroblasts; error bars denote standard deviation; asterisks denote significant difference vs. control (p<0.05). Fig 8E: Example of morphology in PS1-knockdown (shRNA) (>75% knockdown; right panel) and mismatch control (left panel) MEFs. Note "perinuclear" phenotype in PS1-knockdown cells. 63x. Fig. 8F: Quantitation as in (B).

**Fig 9.** ApoE and APP are enriched in MAM. T, Total cellular protein; CM, crude mitochondria; PM, plasma membrane.

**Fig 10.** Western blot analysis of subcellular fractions of mouse liver. Localization and molecular masses of the indicated polypeptides were determined using the antibodies listed at right. Thirty µg of protein were loaded into each lane.

**Fig 11.** Immunolocalization of PEMT in human fibroblasts (Fig 11A) Fixation with PF and permeabilization with TX100. Note poor co-localization of the two signals (the orange staining in the merge panel is the non-specific overlap of the MTred stain with the diffuse anti-PS1 stain). Fig 11B: Fixation and permeabilization with MeOH. Note co-localization of PEMT and MTred in the perinuclear region (yellow arrowheads) but not in more distal regions (red arrowheads). Images captured by confocal microscopy (100x).

**Figure 12.** Immunolocalization of PS1 (C-terminal antibody; Sigma P7854) in mouse 3T3 cells (upper and middle panels) and in human fibroblasts (lower panels). Fig 12A: Fixation in PF and permeabilization in TX100. Fig 12B: Fixation in PF and permeabilization in MeOH. Fig 12C: Fixation and permeabilization in MeOH. Arrowheads as in Fig. 12A. Note similarity of the co-localization pattern to that with PEMT in Fig. 12A. Note also that the similarity of the results in (b) and (c) imply that it is the TX100, not the PF, that is responsible for the diffuse pattern of immunostain shown in (a). 63x

**Figure 13.** Immunolocalization of PEMT and PS1 in human fibroblasts (Fig. 13A) Fixation with PF and permeabilization with TX100. Fig 13B: Fixation and permeabilization with MeOH. Note the high degree of colocalization of the two signals in both sets of images. Images captured by confocal microscopy (100x).
Figure 14. Immunohistochemistry to detect PS1 is various cells. Cells were stained with MTred (red) and with anti-PS1 (green); merged photos are at light (yellow if MTred and PS1 are co-localized). Cells were fixed and permeabilized with MeOH. Fig. 14A: Mouse 3T3 cells immunostained with Ab P4985 that detects the N-terminus of PS1. Fig. 14B: Rat neurons immunostained with Ab P7854 that detects the C-terminus of PS1. Note the co-localization PS1 with the MTred signal, mainly in mitochondria located proximal to the nucleus (yellow arrowheads); there is a lower degree of co-localization in more distal mitochondria (red arrowheads). Immunostaining of P7854 was suppressed in the presence of the peptide epitope used to generate the antibody, confirming its specificity. Fig. 14C: Human 293T cells immunostained with Ab P7854, photographed in a plane of focus to reveal the localization of PS1 to adherens junctions in confluent cells (arrowheads). Note absence of co-localization of PS1 with MTred in adherens junctions. 63x

Figure 15. Western blot analysis of the subcellular fractions of interest (ER-MAM, mitochondria, and ER) from mouse liver and brain. Fig 15A: Thirty µg of total liver protein were loaded in each lane, and were probed using the indicated marker antibodies (at right; approximate mass in parentheses) and various PS1 antibodies (at left). SSR1, signal sequence receptor a; CANX, calnexin; NDUFA9, subunit of mitochondrial respiratory complex I. Fig. 15B: Same as in (A), using brain. Fig. 15C: Relative abundance of each fraction, as determined by Bradford protein assay; the approximate averages are also indicated below each lane in (A) and (B).

Figure 16. Co-localization of MTred, calnexin, and PS1 (antibody P7854) in human fibroblasts, viewed by confocal microscopy (63x). Regions a, b, and c within ovals are discussed in the text.

Figure 17. Mitochondrial morphology in mouse embryonic fibroblasts deficient in PS1 due to sh-RNA treatment. Center. Western blot analysis of shRNA clones. Lanes 1-3, dilutions to quantitate PS1; lane 4, knockdown of PS1 compared to control in lane 5. Anti-tubulin loading controls at bottom. Side panel. MTred staining of test (left) vs. control (right) cells. Note "perinuclear" phenotype in PS1-knockdown cells. The specificity of the shRNA primer was confirmed by transducing a mismatch shRNA.

Figure 18. Mitochondria are more perinuclear in PS1 fibroblasts than in controls. Red, mitochondria; green, microtubules.

Figure 19. gamma-Secretase activity of mouse liver and brain fractions.
[0043] **Figure 20.** Mitochondrial dynamics in PS1-knockdown neuroblastoma cells. Note the severely reduced accumulation of mitochondria in varicosities and at branch points (arrowheads) in cell processes in PS1-KD vs. control cells. In the enlargements in the center panels, mitochondria were enriched in "varicosities" (arrowheads) or uniformly distributed (brackets) in neuronal processes of control cells, but were markedly reduced in numbers, density, and intensity in PS1-KD cells. Quantification of MT Red staining (plots of intensity vs. length by Image J) in each process is at the right of the respective enlargements (note corresponding regions marked a-d). The plots are shown for illustrative purposes only, as they have different intensity scales and are not comparable quantitatively.

[0044] **Figure 21.** Mitochondria in the hippocampal CA1 region of an FAD<sup>PSI</sup> patient (A434C). Immunohistochemistry to detect the FeS subunit of complex III. Note perinuclear "rings" of mitochondria (arrowheads) and the dearth of mitochondria in the distal parts of the cell body (asterisks) in patient vs. control.

[0045] **Figure 22.** Western blot of selected mitochondrial proteins. Rieske and Core B are subunits of complex III of the respiratory chain.

[0046] **Figure 23.** PS1-mutant mouse MEFs have increased ROS. MitoSox staining is increased in both single- and double-KO cells.

[0047] **Figure 24.** Bioenergetics. Fig 24A: Oxygen consumption. Fig. 24B: ATP synthesis.

[0048] **Figure 25.** Ca<sup>2+</sup> homeostasis in control and PS1-knockdown cells. Fig. 25A: Cytosolic Ca<sup>2+</sup> using fura-2 measured at 340/380 nm (example in inset). Note increase (vertical arrow) and delayed release (horizontal arrows) of Ca<sup>2+</sup> upon ATP addition. Fig. 25B: Mitochondrial Ca<sup>2+</sup> using pericams. Blue cells indicate elevated Ca<sup>2+</sup> (example in inset). Note higher [Ca<sup>2+</sup>] in PS1-KD cells. F/F0, ratio of fluorescence at time x to that at time 0.

[0049] **Figure 26.** Mitochondrial morphology in PACS2-KO mice. MT Red (red) and microtubule (green) staining of wt and KO MEFs. Note perinuclear distribution of mitochondria, and shape changes ("doughnuts" in enlargement of boxed region) in the KO cells.

[0050] **Figure 27.** Analysis of PS1 and Ab in mouse brain cell fractions. Fig 27A. Schematic of fractions associated with ER, MAM, and mitochondria. Fig 27B. Western blots
of the indicated fractions (15 mg loaded in each lane), using the indicated antibodies. Note concentration of PS1 in MAM, whereas Ab appears to be concentrated in those mitochondria that are associated with ER ("MER"); notably, neither PS1 nor Ab are associated with "free" mitochondria.

[0051] **Figure 28.** Western blot analysis of subcellular fractions of mouse brain. Thirty µg of total protein were loaded in each lane. Fig 28A: Localization and predicted molecular masses of the indicated polypeptides were determined using the antibodies listed at right (see text). PM, plasma membrane. Fig 28B: Fractions were probed using the indicated antibodies against PS1 (Calbiochem PC267) and PS2 (Cell Signalling 2192) and to other components of the γ-secretase complex. In the blots shown here, the intensity of both the PS1 and the PS2 signals in MAM was enriched ~8-fold over that in the ER.

[0052] **Figure 29.** γ-Secretase activity assays. Fig 29A: Activity using a FRET-based assay, in the absence and presence of Compound E, a γ-secretase inhibitor. Serial dilutions of the indicated subcellular fractions from mouse brain were assayed for APP cleavage activity (in arbitrary units^g protein). Bars, SD; asterisk denotes significant difference in MAM compared to the other fractions (P<0.05); n=3 for all fractions. Fig 29B: Activity using Western blotting to detect AICD, in the absence and presence of Compound E. The identity of the lower bands in the first and third lanes is unknown. The specificity of the AICD signal was confirmed in PS1/PS2 double-knockout mouse embryonic fibroblasts.

[0053] **Figure 30.** Immunocytochemistry to detect FACL4 and presenilins in mammalian cells. Fig. 30A: Double-staining of human fibroblasts with MT Red and anti-FACL4. FACL4 co-localizes with MT Red in regions proximal to the nucleus (yellow arrowhead), with a lower degrees of co-localization in more distal mitochondria (red arrowhead). In an enlarged view of the perinuclear region from another merged field (rightmost panel), note discrete regions where the red and green signals (e.g. arrowheads) are in apposition and do not overlap. Fig. 30B: Double-staining of human fibroblasts with MT Red and anti-PS1. Note the similarity of the co-localization pattern to that seen with FACL4. Fig. 30C: Double-staining of human fibroblasts with anti-FACL4 (red) and anti-PS1 (green). There is significant overlap between the red and green signals, even in the enlarged merged view of the perinuclear region, implying that both proteins are in the same compartment (i.e. MAM). Fig. 30D: Double-staining of mouse 3T3 cells with MT Red and anti-PS2. Note the similarity of the co-localization pattern to that seen in panels A and B. Fig. 30E: Double-
staining of confluent COS-7 cells human with MT Red and anti-PS1, photographed in a plane of focus to reveal the localization of PS1 to adherens junctions (AJ; arrowheads). The MT Red staining is fuzzy because almost all mitochondria are below the plane of focus. Note the absence of co-localization of PS1 with MT Red in AJ. Immunostaining of anti-PS1 (Ab P7854) was suppressed in the presence of the peptide epitope used to generate the antibody, confirming its specificity.

[0054] Figure 31. Phospholipid biosynthetic pathways.

[0055] Figure 32. Incorporation of 3H-Ser into phospholipids. Fig. 32A: Time course (0, 2, 4, 6 hours) of phospholipid synthesis in PS1+PS2 double knockout mouse embryonic fibroblasts (MEFs; courtesy of Bart de Strooper; Herreman et al. (1999) Proc. Natl. Acad. Sci. USA 96:1 1782), in medium lacking Etn and Ser. Note the increase in PtdSer and PtdEtn (and also PtdCho) in DKO MEFs vs. control MEFS. Fig32B: Same as in A, but using a different source of MEFs, from Alan Bernstein (Donoviel et al. (1999) Genes Dev. 13:2801). Fig 32C: Time course (0, 1, 3 hours) of phospholipid synthesis in MEFs were null for PACS2, a gene required for the transport of proteins from the ER across the MAM to mitochondria (Simmen et al. (2005) EMBO J 24:717; a gift of Gary Thomas). As such, PACS2 KO cells should be defective in MAM transport to mitochondria. Note the increase in PtdSer in PACS2-KO MEFS, but a decrease in PtdEtn and PtdCho, consistent with loss of MAM-mitochondrial communication. Fig32D: Fibroblasts from a FAD patient with a mutation in PS1 (A246E) and from PS1-KO MEFs were treated with 3H-Ser for 30 min at 37°C and the ratio of PtdEtn/PtdSer was measured.

[0056] Figure 33. Cholesterol content. Fig. 33A: Free and esterified cholesterol in mouse brain fractions. Fig. 33B: Free and esterified cholesterol in the crude mitochondrial fraction (essentially mitochondria + MAM) from WT and PS1-knock-in mice.

[0057] Figure 34. Mitochondrial dynamics in PS1-knockdown (PS1-KD) neuroblastoma cells. Note the severely reduced accumulation of mitochondria in varicosities and at branch points (arrowheads) in cell processes in PS1-KD vs. control cells. In the enlargements at right (from other cells not shown here), mitochondria were enriched in "varicosities" (arrowheads) or uniformly distributed (brackets) in neuronal processes of control cells, but were markedly reduced in numbers, density, and intensity in PS1-KD cells.

[0058] Figure 35. Mitochondria in the hippocampal CA1 region of an FADPS1 patient (A434C). Immunohistochemistry (FeS subunit of complex III) to detect mitochondria.
Note perinuclear "rings" of mitochondria (arrowheads) and the dearth of mitochondria in the distal parts of the cell body (asterisks) in patient vs. control. Left, low power; right, four neurons (a-d) at higher magnification.

[0059] Figure 36. ApoE and APP are enriched in MAM. PM, plasma membrane.

[0060] Figure 37. Western blot to detect the indicated proteins in standard subcellular fractionation of mouse tissues to isolate PM, MAM, mitochondria, and ER fractions (13). Na/K-ATPase and panchaderhin are enriched in the PM; ACAT1 is enriched in the MAM. Note that the MAM fraction is essentially devoid of the PM marker.

[0061] Figure 38. MAM displays the features of a lipid raft. Fig. 38A: Mouse liver Percoll-purified MAM treated with or without TX100 prior to centrifugation through a second Percoll gradient. The low density fraction (arrow) is detergent-resistant but solubilizable by methanol (MeOH), implying that it is a DRM. Fig. 38B: Western blot of fractions (as in Fig. 38A) from a 5%-30% sucrose gradient (triangle; lower density at left). The pellet (P) denotes TX100-soluble material. Fig. 38C: Western blot of gradient fractions of mouse liver PM and crude mitochondrial extract (CM) to detect Src (PM marker) and Pemt (MAM marker).

[0062] Figure 39. Cholesterol metabolism in normal mouse brain (A, B) and in presenilin-mutant cells (CF). Fig. 39A: Total cholesterol (n=3, except PM rafts [n=2]). Fig. 39B: ACAT1 activity (n=4). Inset: Western blot to detect ACAT1; 20 µg protein loaded in each lane. Figs. 39C-F: Cholesterol metabolism in presenilin-mutant MEFs. Fig. 39C: Content of cholesterol species. Numbers denote ~ng/µg protein. Fig. 39D: EM of a DKO MEF. Note accumulation of lipid droplets (asterisks). M, mitochondrion. Fig. 39E: ACAT activity in MEFs after 6 h incubation with 3H-oleoyl-CoA (n=3). Fig. 39F: ACAT activity in isolated MAM after 20 min incubation (n=4). Fig. 39G: Kinetics of CE synthesis (performed as in (D)) in Psl-KD cells (note increased slope [line of best fit, in cpm^g/h] vs control). Fig. 39H: Quantitation of 3H-CE synthesis after 6h in FAD (n=4) and SAD (n=5) fibroblasts vs paired controls. Error bars, SD; asterisks denote significant difference (p<0.05). Fig. 39I: ACAT activity (i.e. conversion of 3H-cholesterol to 3H-cholesteryl esters) in MEFs after 6 h (n=6).

[0063] Figure 40. Western blot to detect SSRa (signal sequence receptor a; a marker for bulk ER) and NDUFA9 (a subunit of complex I of the respiratory chain; a marker for mitochondria) in fractions from a 5%-30% sucrose gradient (triangle denotes increasing
density from left to right) of purified bulk ER and mitochondria after treatment with 1% Triton X-100 at 4°C for 1 hour. Note that neither bulk ER nor mitochondria contain low density DRMs; almost all of both fractions, as determined by the marker proteins, was in the detergent-soluble pellet (P).

[0064] Figure 41. Phospholipid synthesis in MEFs. Fig. 41A: Synthesis of PtdSer and PtdEtn after 3H-Ser labeling for the indicated times (hours) (n=3). Fig. 41B: Pulse-chase. MEFs labeled for 1 h with 3H-Ser; chase with cold Ser (n=3). Fig. 41C: Phospholipid synthesis in crude mitochondria (n=3 or 4) Error bars, SE; p<0.05. Fig. 41D: Cinnamycin sensitivity in MEFs. Left: Live/dead assay (1 µM cinnamycin for 10 min at 37°C). Right: Cinnamycin-sensitivity assay (10 min at 37°C). Fig. 41E: Kinetics of PtdSer and PtdEtn synthesis (as in (A)) in Psl-KD cells. Fig. 41F: Phospholipid synthesis after 6h (as in (A)) in FAD (n=10) and SAD (n=4) patient fibroblasts.

[0065] Figure 42. Electron microscopy of WT (Fig. 42 B and D) and DKO (Fig. 42A, C, E) MEFs. Note increased length of regions of contact between ER and mitochondria (M) (arrowheads) in DKO MEFs, and ER "sandwiched" between two mitochondria (Fig. 42E). Fig. 42F: Quantitation of ER-mitochondrial contact lengths.

[0066] Figure 43. Cinnamycin sensitivity in fibroblasts from FAD patients with pathogenic mutations in PSI (PSI) and from sporadic AD (SAD) patients. Cells were treated with 1 µM cinnamycin for 10 min at 37°C, and viability was monitored by Live/Dead assay. Note especially the cinnamycin sensitivity in the SAD patients, who presumably have normal PSI expression and function.

[0067] Figure 44. Detection of lipid droplets in PS-mutant cells. Fig. 44A: EM of DKO MEFs. Note the presence of features reminiscent of lipid droplets (asterisks); these were not observed in WT MEFs. M, mitochondrion. Fig. 44B: Left: LipidTox staining of DKO MEFs; note punctate staining (i.e. lipid droplets) that were absent in WT MEFs. Right: Quantitation of LipidTox staining. Fig. 44C: Left: LipidTox staining of Psl-KD cells. Right: Quantitation, as in (B). Note that overexpression of human WT-PS1, but not A246E mutant PS1, reduced lipid droplet formation. C, mismatched shRNA control. Fig. 44D: Example of LipidTox staining (left) and quantitation of LipidTox-positive cells of FAD (n=5) and SAD (n=7) fibroblasts vs controls (C; n=6) (right). See other examples in Figure 49. Other notation as in Figure 39.
Figure 45. Cinnamycin sensitivity in PS-mutant cells. Fig. 45A: Left: Example of live/dead assays (1 µM cinnamycin for 10 min at 37°C). Right: Cinnamycin-sensitivity assay (10 min at 37°C) in PS-mutant MEFs. Fig. 45B: Left: Example of cinnamycin sensitivity in Psl-KD cells vs mismatch control (C). Note that overexpression of human WT PS1 could "rescue" Cin-sensitivity, whereas overexpression of A246E mutant human PS1 could not. Right: Cin-sensitivity in AD patient fibroblasts. Fig. 45C: Left: Example of staining of control and AD patient cells with fluorescent cinnamycin (FL-SA-Ro; orange); cells were counterstained with calcein (green) to visualize overall cell morphology. Right: Quantitation of FL-SA-Ro staining in control (n=5), FAD (n=5), and SAD (n=7) patient fibroblasts. See other examples in Figure 50. Other notation as in Fig. 39.

Figure 46. Analysis of MAM function in Mfn2-KO MEFs. Fig. 46A: Kinetics of CE synthesis (performed as in Figure 2D) in Mfh2-KO MEFs (note decreased slope vs control). Fig. 46B: Phospholipid synthesis after 6 h (as in Figure 4A) in Mfn2-KO cells (right) (n=3). Fig. 46C: Western blot to detect APP and its C-terminal cleavage products C99 and AICD (cleavage scheme at top) in PS-DKO and Mfh2-KO MEFs. Note the absence of AICD in DKO MEFs, and the shift in the ratio of C99:AICD in Mfn2-KO vs WT MEFs. Fig. 46D: Subcellular distribution of presenilins in Mfn2-KO vs WT MEFs. Note that loss of Mfn2 does not alter the predominant localization of presenilins in MAM.

Figure 47. Description of cell line used herein. NA, not applicable; +, experiment performed.

Figure 48. Examples of Oil Red O staining of the indicated cells. Note that the increase in punctate staining in the mouse PSI-KD MEFs was rescued by overexpression of human WT-PS1 but not by A246E mutant PS1.

Figure 49. Figs. 49A-B show examples of LipidTox staining of the indicated cells. Note that the increase in punctate staining in the mouse PSI-KD MEFs was rescued by overexpression of human WT-PS1 but not by A246E mutant PS1.

Figure 50. Examples of staining of AD fibroblasts with fluorescent cinnamycin (FL-SA-Ro). To visualize overall cell morphology, cells were counterstained with calcein.
The issued patents, applications, and other publications that are cited herein are hereby incorporated by reference to the same extent as if each was specifically and individually indicated to be incorporated by reference.

Definitions

The singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

The term "about" is used herein to mean approximately, in the region of, roughly, or around. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth. In general, the term "about" is used herein to modify a numerical value above and below the stated value by a variance of 20%.

As used herein, the term "presenilin" refers to the family of related multi-pass transmembrane proteins that can function as a part of the γ-secretase protease complex. The term presenilin includes presenilin-1 (PS1) and presenilin-2 (PS2). There are at least 7 members of the presenilin family in humans including: PS1 (gene PSEN1; Chr 14q24.2), PS2 (gene PSEN2; Chr 1q42.13), PSL1 (gene SPPL2B; Chr 19p13.3), PSL2 (gene SPPL2A Chr 15q21.2; thought to be in endosomes), PSL3 (gene HM13; Chr 20q1 1.21), PSL4 (gene SPPL3, Chr 12q24.31), PSL5 (gene IMP5; Chr 17q21.31; no introns)

The present invention provides methods that are useful the diagnosis of AD in a subject and methods useful for the identification of compounds or therapeutic agents for treating AD. The methods of the present invention pertain in part to the correlation of AD with abnormal or altered endoplasmic reticulum-mitochondrial-associated membrane (ER-MAM) integrity. As used herein, "altered ER-MAM integrity" or "abnormal ER-MAM integrity" are used interchangeably, and can refer to any condition or state, including those that accompany AD, where any structure or activity that is directly or indirectly related to a ER-MAM function has been changed relative to a control or standard.

In one aspect, abnormal or altered ER-MAM integrity in AD cells is reflected by an increase in communication between the ER and mitochondria in the cell as compared to non-AD cells, or altered ER-MAM structure, or cholesterol content in ER-MAM in the cell as compared to non-AD cells. Without being bound by theory, it is believed that abnormal or altered ER-MAM content or structure causes a multitude of downstream effects, which downstream effects themselves can be correlated with AD. Additionally, abnormal or
altered ER-MAM can be caused by upstream effects that are correlated with AD. Such upstream and downstream effects that correlate with abnormal or altered ER-MAM levels or structure can be considered indicators of altered ER-MAM integrity.

[0080] Thus, in various aspects, an indicator of altered ER-MAM integrity can be any detectable parameter that directly or indirectly relates to a condition, process, or other activity involving ER-MAM and that permits detection of altered or abnormal ER-MAM function or state (as compared to ER-MAM from normal or non-AD cells) in a biological sample from a subject or biological source (detection can also be in the subject or animal model). Exemplary indicators of altered ER-MAM integrity can be, for example, a functional activity or expression level of an ER-MAM-associated protein, subcellular localization of an ER-MAM-associated protein, mitochondrial morphology in a cell, mitochondrial localization in a cell, communication between the ER and mitochondria in a cell, structure of ER-MAM in a cell as reflected by cholesterol content in the ER-MAM, in situ morphology of ER-MAM in a cell, or other criteria as provided herein. In addition, the present methods can involve one or more of the above-mentioned indicators of altered ER-MAM integrity in combination with one or more general indicators of AD. General indicators of AD include, but are not limited to, altered APP processing, amyloid toxicity, tau hyperphosphorylation, altered lipid and cholesterol metabolism, altered glucose metabolism, aberrant calcium homeostasis, glutamate excitotoxicity, inflammation and mitochondrial dysfunction.

[0081] In one aspect, the invention provides a method for diagnosing Alzheimer's disease, the method comprising: (a) obtaining one or more cells from a subject suspected of having Alzheimer's disease, and (b) testing the cells from (a) for one or more indicators of altered ER-MAM integrity.

[0082] In one embodiment, an indicator of altered ER-MAM integrity in an AD cell, or a cell from a subject suspected for having AD is any of: (a) an increased ratio of perinuclear to non-perinuclear mitochondria as compared to a normal control cell; (b) an increased ratio of punctate mitochondria to non-punctate mitochondria as compared to a normal control cell; (c) a decreased amount of mitochondria at extremities of cells as compared to a normal control cell; (d) a decrease in mitochondrial movement as compared to a normal control cell; (e) an increased amount of free radial production as compared to a normal control cell; (f) an increased amount of phosphatidylyserine conversion to phosphatidyethanolamine as compared to a normal control cell; (g) increased mitochondrial calcium levels as compared to a normal control cell; (h) increased cytosolic calcium levels as
compared to a normal control cell; (i) an altered localization of ER-MAM associated proteins to ER-MAM as compared to a normal control cell; (j) altered total ER-MAM as compared to a normal control cell; (k) an altered activity of one or more non-presenilin ER-MAM association proteins as compared to a normal control cell; (l) a decreased amount of functional presenilin in ER-MAM as compared to a normal control cell; (m) an altered amount of association between ER-MAM associated proteins as compared to a normal control cell; (n) an increased sensitivity to cinnamycin induced cell death as compared to a normal control cell; (o) an altered cholesterol content in ER-MAM as compared to a normal control cell; (p) decreased ATP biosynthesis in mitochondria as compared to a normal control cell; (q) an increased communication between ER and mitochondria as compared to a normal control cell; (r) altered glucose metabolism as compared to a normal control cell; (t) an increased amount cell death in response to an apoptogen as compared to a normal control cell; (s) increased ER-MAM function as compared to a normal control cell; (t) increased ACATI activity as compared to a normal control cell; (u) increased cholesterol ester synthesis as compared to a normal control cell; or (v) increased lipid droplet formation as compared to a normal control cell.

[0083] In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising obtaining one or more cells from an individual suspected of having Alzheimer's disease, measuring the ratio of perinuclear mitochondria to non-perinuclear mitochondria in the cell, and comparing the ratio of perinuclear mitochondria to non-perinuclear mitochondria measured in the cell to the ratio of perinuclear mitochondria to non-perinuclear mitochondria measured in a control cell wherein, a greater ratio of perinuclear mitochondria to non-perinuclear mitochondria measured in the cell compared to the control cell indicates that the subject has Alzheimer's disease.

[0084] In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining one or more cells from an individual suspected of having Alzheimer's disease, (b) measuring the ratio of punctate mitochondria to non-punctate mitochondria in the cell of step (a), and (c) comparing the ratio of punctate mitochondria to non-punctate mitochondria measured in the cell of step (a) to the ratio of punctate mitochondria to non-punctate mitochondria measured in a control cell wherein, a greater ratio of punctate mitochondria to non-punctate mitochondria measured in the cell of step (a) compared to the control cell indicates that the subject has Alzheimer's disease.
In another aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining one or more cells from an individual suspected of having Alzheimer's disease, (b) measuring the amount of mitochondria at the extremities of the cell of step (a), and (c) comparing the amount of mitochondria at the extremities measured in the cell of step (a) to the amount of mitochondria at the extremities in a control cell wherein, a reduced ratio of punctate amount of mitochondria at the extremities measured in the cell of step (a) compared to the control cell indicates that the subject has Alzheimer's disease.

Thus, in one aspect, the invention described herein provides a method for diagnosing Alzheimer's disease in a subject by comparing mitochondrial movement in a test cell to mitochondrial movement in a control cell, wherein a reduced amount of mitochondrial movement in a control cell to the test cell indicated that the subject has Alzheimer's disease, the method comprising: (a) obtaining a cell from an individual suspected of having Alzheimer's disease, (b) measuring an amount of mitochondrial movement in the cell step (a), and (c) comparing the amount of mitochondrial movement measured in the cell of step (a) to the amount of mitochondrial movement measured in a control cell wherein, a reduced amount of mitochondrial movement measured in the cell of step (a) compared to the control cell indicates that the subject has Alzheimer's disease.

In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a cell from an individual suspected of having Alzheimer's disease, (b) measuring an amount reactive oxygen species in the cell step (a), and (c) comparing the amount reactive oxygen species measured in the cell of step (a) to the amount reactive oxygen species measured in a control cell wherein, a greater amount reactive oxygen species measured in the cell of step (a) compared to the control cell indicates that the subject has Alzheimer's disease.

In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a cell from an individual suspected of having Alzheimer's disease, (b) measuring an amount of phosphatidylserine conversion to phosphatidylethanolamine in the cell step (a), and (c) comparing the amount of phosphatidylserine conversion to phosphatidylethanolamine measured in the cell of step (a) to the amount of phosphatidylserine conversion to phosphatidylethanolamine measured in a control cell wherein, a greater amount of phosphatidylserine conversion to
phosphatidylethanolamine measured in the cell of step (a) compared to the control cell indicates that the subject has Alzheimer's disease.

[0089] In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample from an individual suspected of having Alzheimer's disease, (b) measuring mitochondrial calcium concentration in the biological sample of step (a), and (c) comparing the mitochondrial calcium concentration measured in the biological sample of step (a) to the mitochondrial calcium concentration measured in a control biological sample wherein, a greater mitochondrial calcium concentration measured in the biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease.

[0090] In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample from an individual suspected of having Alzheimer's disease, (b) measuring cytosolic calcium concentration in the biological sample of step (a), and (c) comparing the cytosolic calcium concentration measured in the biological sample of step (a) to the cytosolic calcium concentration measured in a control biological sample wherein, a greater cytosolic calcium concentration measured in the biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease.

[0091] In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample from an individual suspected of having Alzheimer's disease, (b) measuring an amount of ER-MAM localized presenilin in the biological sample of step (a), and (c) comparing the amount of ER-MAM localized presenilin measured in the biological sample of step (a) to the amount of ER-MAM localized presenilin measured in a control biological sample, wherein an altered amount of ER-MAM localized presenilin measured in the biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease.

[0092] In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample from an individual suspected of having Alzheimer's disease, (b) measuring a total amount of ER-MAM in the biological sample of step (a), and (c) comparing the total amount of ER-MAM measured in the biological sample of step (a) to the total amount of ER-MAM measured in a control biological sample, wherein an altered total amount of ER-MAM measured in the
biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease.

[0093] In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample from an individual suspected of having Alzheimer's disease, (b) measuring an activity of one or more non-presenilin ER-MAM-associated proteins in the biological sample of step (a), and (c) comparing the activity of one or more non-presenilin ER-MAM-associated proteins measured in the biological sample of step (a) to the activity of one or more non-presenilin ER-MAM-associated proteins measured in a control biological sample, wherein an altered amount of activity of one or more non-presenilin ER-MAM-associated proteins measured in the biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease.

[0094] In one aspect, the invention described herein provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: obtaining a biological sample from an individual suspected of having Alzheimer's disease, measuring the amount of functional presenilin in ER-MAM in the biological sample and comparing the amount of functional presenilin in ER-MAM measured in the biological sample to the amount of functional presenilin in ER-MAM measured in a control cell wherein, an decrease amount of functional presenilin in ER-MAM measured in the control cell indicates that the subject has Alzheimer's disease.

[0095] In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample from an individual suspected of having Alzheimer's disease, (b) measuring an amount of an association between one or more ER-MAM-associated proteins in the biological sample of step (a), and (c) comparing the amount of an association between one or more ER-MAM-associated proteins measured in the biological sample of step (a) to the amount of an association between one or more ER-MAM-associated proteins measured in a control biological sample wherein, an altered amount of an association between one or more ER-MAM-associated proteins measured in the biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease.

[0096] the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample from an individual
suspected of having Alzheimer's disease, (b) measuring sensitivity to cinnamycin induced cell death in the biological sample of step (a), and (c) comparing the sensitivity to cinnamycin induced cell death measured in the biological sample of step (a) to sensitivity to cinnamycin induced cell death measured in a control biological sample wherein, an increased sensitivity to cinnamycin induced cell death measured in the biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease.

[0097] In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample from an individual suspected of having Alzheimer's disease, (b) measuring the amount of cholesterol content in ER-MAM in the biological sample of step (a), and (c) comparing the amount of cholesterol content in ER-MAM measured in the biological sample of step (a) to the amount of cholesterol content in ER-MAM measured in a control biological sample wherein, an altered amount of cholesterol content in ER-MAM measured in the biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease.

[0098] In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample from an individual suspected of having Alzheimer's disease, (b) measuring the amount of ATP biosynthesis in mitochondria in the biological sample of step (a), and (c) comparing the amount of ATP biosynthesis in mitochondria measured in the biological sample of step (a) to the amount of ATP biosynthesis in mitochondria measured in a control biological sample wherein, a reduced amount of ATP biosynthesis in mitochondria measured in the biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease.

[0099] In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample from an individual suspected of having Alzheimer's disease, (b) measuring the amount of communication between ER and mitochondria in the biological sample of step (a), and (c) comparing the amount of communication between ER and mitochondria measured in the biological sample of step (a) to the amount of communication between ER and mitochondria measured in a control biological sample wherein, a greater amount of communication between ER and mitochondria measured in the biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease.
In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample from an individual suspected of having Alzheimer's disease, (b) measuring the amount of glucose metabolism in the biological sample of step (a), and (c) comparing the amount of glucose metabolism measured in the biological sample of step (a) to the amount of glucose metabolism measured in a control biological sample wherein, a reduced amount of glucose metabolism measured in the biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease.

In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample from an individual suspected of having Alzheimer's disease, (b) measuring the amount of cell death in response to an apoptogen in the biological sample of step (a), and (c) comparing the amount of cell death in response to an apoptogen measured in the biological sample of step (a) to the amount of cell death in response to an apoptogen measured in a control biological sample wherein, a greater amount of cell death in response to an apoptogen measured in the biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease.

In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample from an individual suspected of having Alzheimer's disease, (b) measuring the amount of ER-MAM function in the biological sample of step (a), and (c) comparing the amount of ER-MAM function measured in the biological sample of step (a) to the amount of ER-MAM function measured in a control biological sample wherein, a greater amount of ER-MAM function measured in the biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease.

In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample from an individual suspected of having Alzheimer's disease, (b) measuring the amount of ACATI activity in the biological sample of step (a), and (c) comparing the amount of ACATI activity measured in the biological sample of step (a) to the amount of ACATI activity measured in a control biological sample wherein, a greater amount of ACATI activity measured in the biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease.
In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample from an individual suspected of having Alzheimer's disease, (b) measuring the amount of cholesterol ester synthesis in the biological sample of step (a), and (c) comparing the amount of cholesterol ester synthesis measured in the biological sample of step (a) to the amount of cholesterol ester synthesis measured in a control biological sample wherein, a greater amount of cholesterol ester synthesis measured in the biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease.

In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample from an individual suspected of having Alzheimer's disease, (b) measuring the amount of lipid droplet formation in the biological sample of step (a), and (c) comparing the amount of lipid droplet formation measured in the biological sample of step (a) to the amount of lipid droplet formation measured in a control biological sample wherein, a greater amount of lipid droplet formation measured in the biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease.

Alzheimer's disease cells can comprise, but are not limited to, cells with a PSI mutation, cells with a PS2 mutation, cells with an APP mutation, human skin fibroblasts derived from patients carrying FAD-causing presenilin mutations, mouse skin fibroblasts, cultured embryonic primary neurons, and any other cells derived from PSI -knock out transgenic mice (containing null mutation in the PSI gene), cells having AD-linked familial mutations, cells having genetically associated AD allelic variants, cells having sporadic AD, cells having ApoE mutations or cells having mutations associated with sporadic AD. Exemplary AD mutations include, but are not limited to APP V717 I APP V717F, APP V717G, APP A682G, APP K/M670/671N/L , APP A713V, APP A713T, APP E693G, APP T673A, APP N66SD, APP I 716V, APP V715M, PS1 113Δ4, PS1 A79V, PS1 V82L, PS1 V96F, PS1 113Δ4 , PS1 Y115C, PS1 Y115H, PS1 T116N, PS1 P117L, PS1 E120D, PS1 E120K, PS1 E123K, PS1 N135D, PS1 M139, PS1 I M139T, PS1 M139VJ 143F, PS1 1143T, PS1 M461, PS1 I M146L, PS1 M146V, PS1 H163R, PS1 H163Y, PS1 S169P, PS1 S169L, PS1 L171P, PS1 E184D, PS1 G209V, PS1 I213T, PS1 L219P, PS1 A231T, PS1 A231V, PS1 M233T, PS1 L235P, PS1 A246E, PS1 L250S, PS1 A260V, PS1 L262F, PS1 C263R, PS1 P264L, PS1 P267S, PS1 R269G, PS1 R269H, PS1 E273A, PS1 R278T, PS1 E280A, PS1 E280G, PS1 L282R, PS1 A285V, PS1 L286V, PS1 S290C (Δ9), PS1 E318G, PS1 G378E,
PS1 G384A, PS1 L392V, PS1 C410Y, PS1 L424R, PS1 A426P, PS1 P436S, PS1 P436Q, PS2 R62H, PS2 N141I, PS2 V1481, or PS2 M293V. For example, the cells obtained in step (a) can be, but are not limited to, an AD model cell, a neuron, a fibroblast, a skin biopsy, a blood cell (e.g. a lymphocyte), an epithelial cell and cells found in urine sediment. In certain embodiments, the neurodegenerative disease can be, for example, a dementia-related disease.

[00107] In one aspect, the invention provides a method for determining whether a test compound is suitable for treating Alzheimer's disease, the method comprising (a) contacting an Alzheimer's Disease model cell with a test compound and determining whether the test compound improves an indicator of ER-MAM integrity as compared to an Alzheimer's Disease model cell that has not been contacted with the test compound.

[00108] An improvement in one or more indicators of ER-MAM integrity as it relates to a condition of Alzheimer's disease can be any of: (a) a decreased ratio of perinuclear to non-perinuclear mitochondria in cells contacted with a test compound as compared to cells not contacted with the test compound; (b) a decreased ratio of punctate mitochondria to non-punctate mitochondria in cells contacted with the test compound as compared to cells not contacted with the test compound; (c) a increased amount of mitochondria at extremities of cells in cells contacted with the test compound as compared to cells not contacted with the test compound; (d) an increased in mitochondrial movement in cells contacted with a test compound as compared to cells not contacted with the test compound; (e) an increased amount of free radial production in cells contacted with the test compound as compared to cells not contacted with the test compound; (f) an decreased amount of phosphatidylserine conversion to phosphatidylethanolamine in cells contacted with the test compound as compared to cells not contacted with the test compound; (g) an decreased mitochondrial calcium levels in cells contacted with the test compound as compared to cells not contacted with the test compound; (h) an decreased cytosolic calcium levels in cells contacted with the test compound as compared to cells not contacted with the test compound; (i) an increased amount of functional presenilin in ER-MAM in cells contacted with a test compound as compared to cells not contacted with the test compound; (j) an decreased sensitivity to cinnamycin induced cell death in cells contacted with the test compound as compared to cells not contacted with the test compound; (k) increased ATP biosynthesis in mitochondria in cells contacted with the test compound as compared to cells not contacted with the test compound; (l) an decreased communication between ER and mitochondria in cells contacted with the test compound as compared to cells not contacted
with the test compound; in cells contacted with a test compound as compared to cells not contacted with the test compound; (m) a decreased amount cell death in response to an apoptogen in cells contacted with the test compound as compared to cells not contacted with the test compound; (n) decreased ER-MAM function in cells contacted with the test compound as compared to cells not contacted with the test compound; (o) decreased ACATI activity in cells contacted with the test compound as compared to cells not contacted with the test compound; (p) decreased cholesterol ester synthesis in cells contacted with the test compound as compared to cells not contacted with the test compound; or (q) decreased lipid droplet formation in cells contacted with the test compound as compared to cells not contacted with the test compound.

[00109] In one aspect the invention provides a method for testing whether compound can decrease in the ratio of perinuclear mitochondria to non-perinuclear mitochondria in a cell. For example, a suitable test compound may be (but is not limited to) a compound which can reduce the ratio of perinuclear mitochondria to non-perinuclear mitochondria in an AD cell.

[00110] In one aspect the invention provides a method for testing whether compound can decrease the ratio punctate to non-punctate mitochondria in a cell. For example, a suitable test compound may be (but is not limited to) a compound which can reduce ratio of punctate to non-punctate mitochondria in an AD cell.

[00111] In one aspect the invention provides a method for testing whether compound can increase the amount of mitochondria at the extremities in a cell. For example, a suitable test compound may be (but is not limited to) a compound which can increase amount of mitochondria at the extremities in an AD cell.

[00112] In one aspect the invention provides a method for testing whether compound can increase mitochondrial movement in a cell. For example, a suitable test compound may be (but is not limited to) a compound which can increase mitochondrial movement in an AD cell.

[00113] In one aspect the invention provides a method for testing whether compound can decrease the amount of one or more reactive oxygen species in a cell. For example, a suitable test compound may be (but is not limited to) a compound which can reduce the amount of one or more reactive oxygen species in an AD cell.
[00114] In one aspect the invention provides a method for testing whether compound can decrease the conversion of phosphatidylserine to phosphatidylethanolamme in a biological sample. For example, a suitable test compound may be (but is not limited to) a compound which can reduce the conversion of phosphatidylserine to phosphatidylethanolamme in an AD cell.

[00115] In one aspect the invention provides a method for testing whether compound can decrease mitochondrial calcium concentration in a biological sample. For example, a suitable test compound may be (but is not limited to) a compound which can reduce mitochondrial calcium concentration in an AD cell.

[00116] In one aspect the invention provides a method for testing whether compound can decrease cytosolic calcium concentration in a biological sample. For example, a suitable test compound may be (but is not limited to) a compound which can reduce cytosolic calcium concentration in an AD cell.

[00117] In one aspect the invention provides a method for testing whether compound can alter localization of ER-MAM associated proteins to ER-MAM in a biological sample. For example, a suitable test compound may be (but is not limited to) a compound which can alter localization of ER-MAM associated proteins to ER-MAM in an AD cell.

[00118] In one aspect the invention provides a method for testing whether compound can alter the total amount of ER-MAM in a biological sample. For example, a suitable test compound may be (but is not limited to) a compound which can alter the total amount of ER-MAM in an AD cell.

[00119] In one aspect the invention provides a method for testing whether compound can alter the activity of one or more non-presenilin ER-MAM-associated proteins in a biological sample. For example, a suitable test compound may be (but is not limited to) a compound which can alter the activity of one or more non-presenilin ER-MAM-associated proteins in an AD cell.

[00120] In one aspect the invention provides a method for testing whether compound can increase the amount of functional presenilin in ER-MAM in a biological sample. For example, a suitable test compound may be (but is not limited to) a compound which can increase the amount of functional presenilin in ER-MAM in an AD cell.
[00121] In one aspect the invention provides a method for testing whether compound can alter the association of ER-MAM-associated proteins (e.g. Diacylglycerol-O-acyltransferase 2 (DGAT2) and stearoyl-CoA desaturase 1 (SCD1)). For example, a suitable test compound may be (but is not limited to) a compound which can alter the association of ER-MAM-associated proteins (e.g. Diacylglycerol-O-acyltransferase 2 (DGAT2) and stearoyl-CoA desaturase 1 (SCD1)) in an AD cell.

[00122] In one aspect the invention provides a method for testing whether compound can decrease sensitivity to cinnamycin induced cell death. For example, a suitable test compound may be (but is not limited to) a compound which reduce sensitivity to cinnamycin induced cell death of an AD cell contacted with cinnamycin.

[00123] In one aspect the invention provides a method for testing whether compound can alter cholesterol content in ER-MAM in a biological sample. For example, a suitable test compound may be (but is not limited to) a compound which can alter cholesterol content in ER-MAM in an AD cell.

[00124] In one aspect the invention provides a method for testing whether compound can increase ATP biosynthesis in mitochondria M in a biological sample. For example, a suitable test compound may be (but is not limited to) a compound which can increase ATP biosynthesis in mitochondria in an AD cell.

[00125] In one aspect the invention provides a method for testing whether compound can cause an a decrease in the amount of communication between the ER and mitochondria in a biological sample. For example, a suitable test compound may be (but is not limited to) a compound which can reduce communication between the ER and mitochondria in an AD cell.

[00126] In one aspect the invention provides a method for testing whether compound can cause an a decrease in the amount of cell death in response to an apoptogen in a biological sample. For example, a suitable test compound may be (but is not limited to) a compound which can reduce cell death in response to an apoptogen in an AD cell.

[00127] In one aspect the invention provides a method for testing whether compound can alter glucose metabolism in a biological sample. For example, a suitable test compound may be (but is not limited to) a compound which can alter glucose metabolism in an AD cell.
In one aspect the invention provides a method for testing whether compound can cause an a decrease in the amount of ACATI activity in a biological sample. For example, a suitable test compound may be (but is not limited to) a compound which can reduce ACATI activity in an AD cell.

In one aspect the invention provides a method for testing whether compound can cause an a decrease in the amount of cholesterol ester synthesis in a biological sample. For example, a suitable test compound may be (but is not limited to) a compound which can reduce the cholesterol ester synthesis in an AD cell.

In one aspect the invention provides a method for testing whether compound can cause an a decrease in the amount of lipid droplet formation in a biological sample. For example, a suitable test compound may be (but is not limited to) a compound which can reduce lipid droplet formation in an AD cell.

Presenilin-1 (PS1), presenilin-2 (PS2), and γ-secretase activity, which play a role in the pathogenesis of Alzheimer disease (AD), are located in lipid rafts, but most of this activity has been found to reside intracellularly.

In certain aspects, the invention described herein relates to the finding that PS's and γ-secretase activity are enriched in a subcompartment of the endoplasmic reticulum (ER) that is physically and biochemically connected linked to mitochondria, called mitochondria associated ER membranes (MAM).

In certain aspects, the invention described herein relates to the finding that MAM displays the features of an intracellular detergent-resistant membrane.

In certain aspects, the invention described herein relates to the finding that the loss of presenilin function in genetically engineered cells and in fibroblasts from patients with familial and sporadic AD results in increased ER-mitochondrial communication and that this upregulation correlated with a significantly greater area of physical contact between ER and mitochondria.

In certain aspects, the invention described herein relates to the finding that presenilins are negative regulators of ER mitochondrial communication

In certain aspects, the invention described herein relates to the finding that increased cross-talk between ER and mitochondria is found in AD patient cells and can contribute to pathogenesis of the AD.
[00137] In certain aspects, the invention described herein relates to the finding that ER-MAM is a specific subcompartment of the endoplasmic reticulum (ER) that is associated intimately with mitochondria, called ER mitochondria-associated membrane. ER-MAM forms a physical bridge between the two organelles. When ER-MAM integrity is compromised (e.g. by treating cells with methanol or with the pro-apoptotic agent staurosporin), ER-MAM-localized PS1 and PS2, as well as other known ER-MAM localized proteins, such as phosphatidylserine-N-methyltransferase 2 (PEMT; involved in phospholipid metabolism) and acyl-CoA:cholesterol-transferase (ACAT 1; involved in cholesterol metabolism) redistribute to mitochondria located in the perinuclear region (where the ER-MAM is concentrated).

[00138] In AD cells, abnormal or altered ER-MAM integrity can be, for example, an increase in communication between the ER and mitochondria in the cell as compared to non-AD cells, or altered ER-MAM structure, or cholesterol content in ER-MAM in the cell as compared to non-AD cells. As used herein, an amount of communication between the ER and mitochondria can refer to an amount of ER-MAM function or activity. Thus, in certain embodiments described herein, an increase in communication between the ER and mitochondria refers to an increase in ER-MAM function or activity, whereas a decrease in communication between the ER and mitochondria refers to a decrease in ER-MAM function or activity. Without being bound by theory, it is believed that abnormal or altered ER-MAM causes a multitude of downstream effects, which downstream effects themselves can be correlated with AD. Thus, in some embodiments, the present methods comprise the detection or assaying for an increased or decreased level of at least one indicator of altered ER-MAM integrity.

[00139] According to the present invention, an "indicator of altered ER-MAM integrity" can be any detectable parameter that directly or indirectly relates to a condition, process, or other activity involving ER-MAM and that permits detection of altered or abnormal ER-MAM function or state (as compared to ER-MAM from normal or non-AD cells) in a biological sample from a subject or biological source. Detection can also be in the subject or animal model. For example, indicators of altered ER-MAM integrity can be, but are not limited to, a functional activity or expression level of an ER-MAM-associated protein, subcellular localization of an ER-MAM-associated protein, mitochondrial morphology in a cell, mitochondrial localization in a cell, mitochondrial movement in a cell, communication between the ER, and altered ER-MAM structure as reflected by cholesterol content in the
ER-MAM, in situ morphology of ER-MAM in a cell, or other criteria as provided herein. In addition, the present methods can involve one or more of the above-mentioned indicators of altered ER-MAM integrity in combination with one or more general indicators of AD. General indicators of AD include, but are not limited to, altered APP processing, amyloid toxicity, tau hyperphosphorylation, altered lipid and cholesterol metabolism, altered glucose metabolism, aberrant calcium homeostasis, glutamate excitotoxicity, inflammation and mitochondrial dysfunction.

Alzheimer's Disease

[00140] The present invention provides compositions and methods that are useful the diagnosis of Alzheimer's disease in a subject and in the identification of compounds or therapeutic agents for treating Alzheimer's disease.

[00141] Alzheimer disease (AD) is a neurodegenerative dementing disease of relatively long course and late onset. The neuronal loss is especially evident in the cortex and hippocampus. AD, a leading cause of dementia, is one of several disorders that cause the gradual loss of brain cells. Dementia is an umbrella term for several symptoms related to a decline in thinking skills. Symptoms include a gradual loss of memory, problems with reasoning or judgment, disorientation, difficulty in learning, loss of language skills and a decline in the ability to perform routine tasks. People with dementia also experience changes in their personalities and experience agitation, anxiety, delusions, and hallucinations.

[00142] Pathologies of AD include the atrophy of brain gray matter as a result of the massive loss of neurons and synapses, and protein deposition in the form of both intraneuronal neurofibrillary tangles and extracellular amyloid plaques within the brain parenchyma. In addition, affected areas of the AD brain exhibit a reactive gliosis that appears to be a response to brain injury. Surviving neurons from vulnerable populations in AD show signs of metabolic compromise as indicated by alterations in the cytoskeleton (Wang et al., *Nature Med.*, 1996, 2, 871-875), Golgi complex (Salehi et al., *J. Neuropath. Exp. Neurol.*, 1995, 54, 704-709) and the endosomal-lysosomal system (Cataldo et al, *Neuron*, 1995, 14, 671-680).

[00143] Biochemically, the disease is characterized by the appearance of neuritic senile plaques composed of β-amyloid, and neurofibrillary tangles composed of hyperphosphorylated and aggregated Tau proteins. The familial form (FAD) is associated with mutations in amyloid precursor protein (APP), in presenilin 1 (PS1), and in presenilin 2...
(PS2). PS1 and PS2 are aspartyl proteases. They are components of the γ-secretase complex, that cleaves APP within the plasma membrane to ultimately produce amyloid β-peptide. The γ-secretase complex also contains APH1 (with at least 3 isoforms), PEN2, and NCT (nicastrin; also called APH2). Following cleavage of the amyloid precursor protein (APP) by a- and β-secretases, γ-secretase cleaves the remaining APP polypeptide to release small amyloidogenic fragments 40- and 42-aa in length (Aβ40 and Aβ42). These fragments have been implicated in the pathogenesis of AD. Presenilins cleave their target polypeptides within membranes (Wolfe and Kopan, 2004).

The vast majority of AD is sporadic (SAD), but at least five gene loci, and three genes, have been identified in the familial form (FAD). The three genes are amyloid β precursor protein (APP, on chromosome 21q21.3), presenilin 1 (PS1, on 14q24.2), and presenilin 2 (PS2, on 1q42.13).

Presenilins

PS1 and PS2 share an overall 67% amino acid sequence homology. Primary structure analysis indicates they are integral membrane proteins with 6 to 8 transmembrane domains (Slunt et al, Amyloid-Int. J Exp. Clin. Invest., 1995, 2, 188-190; Doan et al, Neuron, 1996, 17, 1023-1030). The presenilin proteins are processed proteolytically through two intracellular pathways. Under normal conditions, accumulation of 30 kDa N-terminal and 20 kDa C-terminal proteolytic fragments occurs in the absence of the full-length protein. This processing pathway is regulated and appears to be relatively slow, accounting for turnover of only a minor fraction of the full-length protein. The remaining fraction is degraded in a second pathway by the proteasome (Thinakaran et al, Neuron, 1996, 17, 181-190; Kim et al, J. Biol. Chem., 1997, 272, 11006-1 1010).

FAD linked to the presenilin mutations is highly penetrant. The aggressive nature of the disease indicates that the mutant protein participates in a seminal pathway of AD pathology. To date, over seventy FAD mutations have been identified in PS1, and three FAD mutations have been found in PS2. Most of the FAD mutations occur in conserved positions between the two presenilin proteins, indicating that they affect functionally or structurally important amino acid residues. All but two of the presenilin mutations are missense mutations. One exception results in an aberrant RNA splicing event that eliminates exon 9, creating an internally-deleted mutant protein (Perez-Tur et al., NeuroReport, 1995, 7, 297-301; Sato et al, Hum. Mutat. Suppl, 1998, 1, S91-94; and Prihar et al, Nature Med.,...
The other results in two deletion transcripts (Δ4 and A4cryptic) and one full-length transcript with the amino acid Thr inserted between 113 and 114 (DeJonghe et al., Hum. Molec. Genet., 1999, 8, 1529-1540). The latter transcript leads to the AD pathophysiology.

Presenilins form the catalytic subunit of the γ-secretase complex that produces the Aβ peptide. Most mutations in APP, PS1 and PS2 result in an increase in the ratio of a 42-residue form of Aβ (Aβ42) versus 40-residue Aβ (Aβ40). Aβ peptides ending at residue 42 or 43 (long tailed Aβ) are more fibrillogenic and more neurotoxic than Aβ ending at residue 40, which is the predominant isoform produced during normal metabolism of βAPP (St. George-Hyslop, P. H., & Petit, A., C. R. Biologies (2004) 328:1 19-130; Selkoe, D. J., J Clin Invest (2002) 110:1375-1381).


PS1 has been localized to numerous regions of the cell, including the plasma membrane (Georgakopoulos et al, 1999; Baki et al, 2001; Marambaud et al, 2002; Marambaud et al, 2003; Tarassishin, 2004), the Golgi (Siman et al, 2003; Kimura et al, 2001), and the endoplasmic reticulum (De Strooper et al, 1997; Wolfe et al, 2004), endosomes/lysosomes, the nuclear envelope (Wolfe et al, 2004), and adherens junctions (Marambaud et al, 2002). PS1 has not been found in mitochondria, except for reports from one group that used Western blotting and immunoelectron microscopy, not immunohistochemistry, to localize PS1 to the rat mitochondrial inner membrane (Ankarcrona et al, 2002; Hansson et al, 2005). Another group used immuno electron microscopy and found PS1 in the ER, in the perinuclear region, and at the plasma membrane (at areas of cell-to-cell contact), but not in mitochondria (Takashima et al, 1996). Using immunoelectron microscopy and Western blotting, APH1, NCT, and PEN2 were found to reside in rat mitochondria (Ankarcrona et al, 2002, Hansson et al, 2004).
ER-MAM and ER-MAM-associated proteins

[00150] The present invention provides compositions and methods that are useful in the diagnosis of neurodegenerative diseases, including Alzheimer's disease, and in the identification of compounds or therapeutic agents for treating neurodegenerative diseases, including dementia, and including Alzheimer's disease.

[00151] ER-MAM is a specific compartment involved in the synthesis and transfer of phospholipids between the ER and mitochondria (Vance (1990) JBC 265:7248). ER-MAM-localized proteins (ER-MAM-associated proteins) are involved in intermediate, sphingolipid, ganglioside, fatty acid, and cholesterol metabolism, as well as in apoptosis and calcium homeostasis (Table 1). ER-MAM can also contain enzymes involved in glycosylphosphatidylinositol synthesis (Rogaeva et al, 2007), the unfolded protein response (Zhou et al, 2007), proteasomal function (De Strooper, 2003; Siman and Velji, 2003), and mitochondrial import (Kaether et al, 2006) and fission (Tarassishin et al, 2004). The microsomal triglyceride transfer protein contains two subunits, a large subunit (MTTP), and a small subunit that has been identified as protein disulfide isomerase (PDI) (Cupers et al, 2001). MTTP is a ER-MAM-associated protein (Kimura et al, 2001), but it is unclear if PDI is also ER-MAM-associated (Vetrivel et al, 2004). Finally, ApoE, which is a secreted protein, is present intracellularly in high abundance in the ER-MAM fraction (Vance (1990) JBC 265:7248).

[00152] As used herein, an "ER-MAM-associated protein" includes, but is not limited to, proteins localized or concentrated in the ER-MAM such as those listed in Table 1.

Table 1. Proteins Localized or Concentrated in ER-MAM

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT1</td>
<td>Protein kinase AKT1 (PKB)</td>
<td>Calcium</td>
<td>Giorgi (2010) Science 330;1247</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------</td>
<td>------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>APOC1</td>
<td>Apolipoprotein C1</td>
<td>Cholesterol metab</td>
<td>Vance (1990) J Biol Chem 265:7248</td>
</tr>
<tr>
<td>APOC3</td>
<td>Apolipoprotein C3</td>
<td>Cholesterol metab</td>
<td>Vance (1990) J Biol Chem 265:7248</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid beta precursor protein</td>
<td>Notch signaling</td>
<td>Described herein</td>
</tr>
<tr>
<td>ATP2A1</td>
<td>SERCA1 exon-11 spliced variant S1T (SERCA1T)</td>
<td>ER stress</td>
<td>Chami (2011) Mol Cell 32:641</td>
</tr>
<tr>
<td>BCAP31</td>
<td>BAP31 (B-cell receptor-associated protein 31)</td>
<td>Pro-apoptotic</td>
<td>Iwasawa (2011) EMBO J 30:556</td>
</tr>
<tr>
<td>BCL2</td>
<td>BCL2</td>
<td>Apoptosis</td>
<td>Meunier (2010) J Pharmacol Exp Ther 332:388</td>
</tr>
<tr>
<td>BSG</td>
<td>Basigin/CD147/EMMPRIN</td>
<td>Regulatory component of g-secretase</td>
<td>Hashimoto (2006) AJPEM 290:E1237</td>
</tr>
<tr>
<td>C1RL</td>
<td>Complement component 1, r subcomponent</td>
<td>Protein maturation</td>
<td>Wicher (2004) PNAS 101:14390</td>
</tr>
<tr>
<td>CALR3</td>
<td>Calreticulin 3</td>
<td>Calcium</td>
<td>Wieckowski (2009) Nat Protocols 11:1582</td>
</tr>
<tr>
<td>FIS1</td>
<td>Fission 1 homologue</td>
<td>Mitochondrial fission</td>
<td>Iwasawa (2011) EMBO J 30:556</td>
</tr>
<tr>
<td>----</td>
<td>----------------------</td>
<td>-------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>HS1BP3</td>
<td>HCLS1-binding protein 3 (HS1 binding protein 3)</td>
<td>Pro-apoptotic</td>
<td>Shi (2011) BMB Reports 44:381</td>
</tr>
<tr>
<td>HSPA9</td>
<td>Glucose-regulated protein 75-kDa (GRP75; Mortalin-2)</td>
<td>Binds VDAC</td>
<td>Szabadkai (2006) JCB 175:901</td>
</tr>
<tr>
<td>ITPR1</td>
<td>IP3 receptor, type 1</td>
<td>Calcium</td>
<td>Szabadkai (2006) J Cell Biol 175:901</td>
</tr>
<tr>
<td>ITPR3</td>
<td>Inositol 1,4,5-triphosphate receptor, type 3 (IP3R3)</td>
<td>Calcium Homeostasis</td>
<td>C. C. Mendes et al., J. Biol Chem. 280,40892 (2005).</td>
</tr>
<tr>
<td>MAVS</td>
<td>MAVS (Mitochondrial antiviral signaling protein)</td>
<td>Immunity</td>
<td>Horner (2011) Proc natl Acad sci USA 108;14590</td>
</tr>
<tr>
<td>MOAT2</td>
<td>Membrane bound O-acyltransferase domain containing 2</td>
<td>Phospholipid synthase</td>
<td>Rieckhof (2007) JBC 282:28344</td>
</tr>
<tr>
<td>MFN2</td>
<td>Mitofusin-2</td>
<td>Other</td>
<td>de Brito et al. (2008) Nature 456:605</td>
</tr>
<tr>
<td>OPPR1</td>
<td>Opioid receptor, sigma 1</td>
<td>Calcium homeostasis</td>
<td>T. Hayashi, T. P. Su, Cell 131,596 (2007).</td>
</tr>
<tr>
<td>PIGN</td>
<td>α1-4 mannosyltransferase Glycolipid metab</td>
<td>Maeda (2001) EMBO J 20:250</td>
<td></td>
</tr>
<tr>
<td>Protein Name</td>
<td>Protein Function</td>
<td>Metabolism/Integrity</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------</td>
<td>----------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PPP2CA</td>
<td>Protein phos. 2A, subunit C</td>
<td>Calcium</td>
<td>Giorgi (2010) Science 330:1247</td>
</tr>
<tr>
<td>PPP2R1A</td>
<td>Protein phos. 2A, subunit Aα</td>
<td>Calcium</td>
<td>Giorgi (2010) Science 330:1247</td>
</tr>
<tr>
<td>PPP2R1B</td>
<td>Protein phos. 2A, subunit Aβ</td>
<td>Calcium</td>
<td>Giorgi (2010) Science 330:1247</td>
</tr>
<tr>
<td>PS1</td>
<td>Presenilin 1</td>
<td>ER-MAM Integrity</td>
<td>As described herein</td>
</tr>
<tr>
<td>PS2</td>
<td>Presenilin 2</td>
<td>ER-MAM Integrity</td>
<td>As described herein</td>
</tr>
<tr>
<td>RTN4</td>
<td>Reticulon 4 (NOGO), isoform B (NOGO-B)</td>
<td>ER tubulation</td>
<td>Sutendra (2011) Science Transl Med 3:383m55</td>
</tr>
<tr>
<td>RYR1</td>
<td>Ryanodine Receptor type 1</td>
<td>Calcium Homeostasis</td>
<td>O. Kupch, I. Kruglikov, T. Pivneva, N. Voitenko, N. Fedirko, Cell Calcium 43:469 (2007).</td>
</tr>
<tr>
<td>RYR2</td>
<td>Ryanodine Receptor type 2</td>
<td>Calcium Homeostasis</td>
<td>O. Kupch, I. Kruglikov, T. Pivneva, N. Voitenko, N. Fedirko, Cell Calcium 43:469 (2007).</td>
</tr>
<tr>
<td>RYR3</td>
<td>Ryanodine Receptor type 3</td>
<td>Calcium Homeostasis</td>
<td>O. Kupch, I. Kruglikov, T. Pivneva, N. Voitenko, N. Fedirko, Cell Calcium 43:469 (2007).</td>
</tr>
<tr>
<td>SHC1</td>
<td>Src homology and collagen</td>
<td>Redox</td>
<td>Lebedzinska (2009) Arch Biochem Biophys 486:73</td>
</tr>
<tr>
<td>SIGMAR1</td>
<td>Sigma-1 type opioid receptor</td>
<td>Calcium</td>
<td>Hayashi (2007) Cell 131:596</td>
</tr>
<tr>
<td>TCHP</td>
<td>Trichoplein keratin filament-binding protein (Mitostatin)</td>
<td>ER-mito tethering</td>
<td>Cerqua (2010) EMBO Rep 11:854</td>
</tr>
</tbody>
</table>
TMEM173 Stimulator of interferon genes (STING) Interacts w MAVS West (2011) Nat Rev Immunol in press; Ishikawa09
VDAC1 Voltage -dependent anion channel 1 (Porin 1) Ion transp Szabádkai et al. (2006) J Cell Biol. 175:901

[00153] Genetic linkage studies have revealed that FAD is heterogeneous and a majority of the cases have been linked to gene mutations on chromosomes 1, 14, 19, or 21 (reviewed in Siman and Scott, *Curr. Opin. Biotech.*, 1996, 7, 601-607). Affected individuals develop the classical symptomatic and pathological profiles of the disease confirming that the mutations are associated with the development of the disease rather than a related syndrome.

[00154] Several proteins close to FAD-linked loci, as assessed by maximum LOD score, have been identified (Table 2).

Table 2: Comparison of AD loci (marker with highest LOD) (Top) to adjacent MAM protein genes (Bottom)

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Locus</th>
<th>inB</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1S218</td>
<td>1q25</td>
<td>172.7</td>
<td>Liu et al. (2007) Am J Hum Genet 81:17</td>
</tr>
<tr>
<td>SOAT1/ACAT1</td>
<td>1q25.2</td>
<td>177.5</td>
<td>Rusinol (1994) JBC 269:27494</td>
</tr>
</tbody>
</table>

| D3S2418 | 3q28  | 193.8 | Lee (et al. 2006) Arch Neurol 63:1591 |
| ST6GAL1/SIAT1 | 3q27.3 | 188.1 | Ardail (2003) Biochem J 371:1013 |
| D6S1051 | 6p21.3 | 36.7 | Lee (et al. 2006) Arch Neurol 63:1591 |
| D7S2847 (DLD locus) | 7q3.1 | 118.5 | Brown et al.(2007) Neurochem Res 32:857 |
| SOAT1/ACAT1 Exon Xa | 7q3.1 | 120.5 | Li (1999) JBC 274 11060 |
| D8S1119 | 8q21.2-21.3 | 87.2 | Gedraitis et al. (2006) JMG 43:93 1 |
| PTDSS1 | 8q22.1 | 97.3 | Stone et al. (2000) J Biol Chem 275:34534 |
In certain embodiments of the invention, an ER-MAM-associated protein is a natural or recombinant protein, polypeptide, an enzyme, a holoenzyme, an enzyme complex, an enzyme subunit, an enzyme fragment, derivative or analog or the like, including a truncated, processed or cleaved enzyme (Enzymol. 260:14; Ernster et al, 1981 J. Cell Biol. 91:227s-255s, and references cited therein).

An ER-MAM-associated protein can optionally include one or more additional components. As a non-limiting example of an optional component, a ER-MAM-associated protein can further comprise a flexible region comprising a flexible spacer. Spacers can be useful to allow conformational flexibility when one or more peptides are joined in the context of a fusion protein (e.g. GFP fusion proteins or epitope tagged proteins).

Non-limiting examples of a flexible spacer include, e.g., a polyglycine spacer or an polylyalanine spacer. A flexible region comprising flexible spacers can be used to adjust the length of a polypeptide region in order to optimize a characteristic, attribute or property of a polypeptide. Such a flexible region is operably-linked in-frame to the ER-MAM-associated protein as a fusion protein. As one non-limiting example, a polypeptide region comprising one or more flexible spacers in tandem can be use to better present a donor fluorophore or acceptor, thereby facilitating the resonance transfer energy of the donor fluorophore and acceptor pair.
An ER-MAM-associated protein further can include, without limitation, one or more of the following: epitope-binding tags, such as, e.g., FLAG, Express.TM., human Influenza virus hemagglutinin (HA), human p62.sup.c-Myc protein (c-MYC), Vesicular Stomatitis Virus Glycoprotein (VSV-G), glycoprotein-D precursor of Herpes simplex virus (HSV), V5, and AUI; affinity-binding, such as, e.g., polyhistidine (HIS), streptavidin binding peptide (strep), and biotin or a biotinylation sequence; peptide-binding regions, such as, e.g., the glutathione binding domain of glutathione-S-transferase, the calmodulin binding domain of the calmodulin binding protein, and the maltose binding domain of the maltose binding protein; immunoglobulin hinge region; an N-hydroxysuccinimide linker; a peptide or peptidomimetic hairpin turn; or a hydrophilic sequence or another component or sequence that, for example, promotes the solubility or stability of the ER-MAM-associated protein. Non-limiting examples of specific protocols for selecting, making and using an appropriate binding peptide are described in, e.g., Epitope Tagging, pp. 17.90-17.93 (Sambrook and Russell, eds., Molecular Cloning A Laboratory Manual, Vol. 3, 3.sup.rd ed. 2001; Antibodies: A Laboratory Manual (Edward Harlow & David Lane, eds., Cold Spring Harbor Laboratory Press, 2.sup.nd ed. 1998; and Using Antibodies: A Laboratory Manual: Portable Protocol No. 1 Edward Harlow & David Lane, Cold Spring Harbor Laboratory Press, 1998), which are hereby incorporated by reference.

In addition, non-limiting examples of binding peptides as well as well-characterized reagents, conditions and protocols are readily available from commercial vendors that include, without limitation, BD Biosciences-Clontech, Palo Alto, Calif; BD Biosciences Pharmingen, San Diego, Calif; Invitrogen, Inc, Carlsbad, Calif; QIAGEN, Inc., Valencia, Calif; and Stratagene, La Jolla, Calif. These protocols are routine procedures well within the scope of one skilled in the art and from the teaching herein.

Indicators of ER-MAM Integrity

The methods of the present invention pertain in part to the correlation of AD with an increased or decreased level of at least one indicator of altered ER-MAM integrity. For example, indicators of altered ER-MAM integrity include, but are not limited to: (1) whether the communication between the ER and mitochondria in FAD<sup>PS1</sup> or FAD<sup>PS2</sup> cells is increased as compared to controls, (2) whether the structure of MAM is altered or the amount of cholesterol in MAMs are increased in cells from subjects with AD, (3) whether mitochondrial distribution is different in fibroblasts between age-matched controls and patients with FAD harboring pathogenic mutations in presenilin, such as whether almost all
the FADPS1 or FADPS2 mitochondria are in the perinuclear region and/or whether fewer
FAD PS1 or FAD PS2 mitochondria are in the extremities of fibroblasts as compared to control,
and (4) whether FADPS1 or FADPS2 mitochondria appear less elongated (e.g. less tubular) and
more "punctate". In some embodiments, present methods further comprise screening for: (1)
elevated cholesterol levels, (2) altered brain glucose metabolism, (3) altered lipid metabolic
profiles, (4) significant increases in PC and PE in sporadic AD patient brains, (5) disturbed
calcium homeostasis as a feature of both SAD and FAD, and/or (6) cells with presenilin
mutations and ApoE3/E4 or ApoE4/E4 genotype. In some embodiments, methods for
screening for AD do not involve any genetic screen for PS1, PS2, or APP mutations.

[00160] In certain embodiments of the present invention, AD can be correlated
with an increased or decreased level of at least one "indicator of altered ER-MAM integrity". An indicator of ER-MAM integrity refers to an indicator of altered ER-MAM function, as provided herein. In some embodiments, an alteration in ER-MAM function can be
determined with at least one indicator of altered ER-MAM integrity.

[00161] Altered ER-MAM integrity can have its origin in extra ER-MAM
structures or events as well as in ER-MAM structures or events, in direct interactions
between ER-MAM-associated proteins and proteins outside of ER-MAM genes or in
structural or functional changes that occur as the result of interactions between intermediates
that can be formed as the result of such interactions, including metabolites, catabolites,
substrates, precursors, cofactors and the like.

[00162] Additionally, altered ER-MAM integrity can include altered metabolic or
other biochemical or biophysical activity in some or all cells of a biological source. As non-
limiting examples, cholesterol metabolism can be related to altered ER-MAM integrity, as
can be generation of phosphatidylethanolamine or defective ER-MAM-associated protein
localization and/or function. As further examples, altered mitochondrial localization, altered
mitochondrial morphology, induction of apoptotic pathways and formation of atypical
chemical and biochemical protein complexes within a cell, whether by enzymatic or non-
enzymatic mechanisms, can be regarded as indicative of altered ER-MAM integrity. These
and other non-limiting examples of altered ER-MAM integrity are described in greater detail
herein.

[00163] In one embodiment, the invention provides a method for diagnosing
Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample
from an individual suspected of having Alzheimer's disease, (b) measuring an amount of an indicator of ER-MAM integrity in the biological sample of step (a), and (c) comparing the amount of the indicator of ER-MAM integrity measured in the biological sample of step (a) to the amount of an indicator of ER-MAM integrity measured in a control biological sample wherein, a reduced amount of the indicator of ER-MAM integrity measured in the biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease.

[00164] In another embodiment, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample from an individual suspected of having Alzheimer's disease, (b) measuring an amount of an indicator of ER-MAM integrity in the biological sample of step (a), and (c) comparing the amount of the indicator of ER-MAM integrity measured in the biological sample of step (a) to the amount of an indicator of ER-MAM integrity measured in a control biological sample wherein, a greater amount of the indicator of ER-MAM integrity measured in the biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease. In another aspect, the present methods for diagnosis can also be used with subjects as a method for predicting whether a subject has a higher probability of becoming afflicted with Alzheimer's disease.

[00165] Thus, an "indicator of altered ER-MAM integrity" can be any detectable parameter that directly or indirectly relates to a condition, process, or other activity involving ER-MAM and that permits detection of altered or abnormal ER-MAM function or state (as compared to ER-MAM from normal or non-AD cells) in a biological sample from a subject or biological source (detection can also be in the subject or animal model).

[00166] Exemplary indicators of altered ER-MAM integrity can be, for example, a functional activity or expression level of an ER-MAM-associated protein, subcellular localization of an ER-MAM-associated protein, mitochondrial morphology in a cell, mitochondrial localization in a cell, communication between the ER and mitochondria, structure of ER-MAM in a cell as reflected by cholesterol content in the ER-, in situ morphology of ER-MAM in a cell, or other criteria as provided herein. In addition, the present methods can involve one or more of the above-mentioned indicators of altered ER-MAM integrity in combination with one or more general indicators of AD. General indicators of AD include, but are not limited to, altered APP processing, amyloid toxicity, tau hyperphosphorylation, altered lipid and cholesterol metabolism, altered glucose metabolism,
aberrant calcium homeostasis, glutamate excitotoxicity, inflammation, mitochondrial
dysfunction, and genetic screens for PS1, PS2, and/or APP mutations associated with AD.

[00167] In another embodiment, the diagnosis can be performed by comparing the
increase or a decrease an indicator of ER-MAM integrity in a test biological sample in
comparison to an indicator of ER-MAM integrity in a control biological sample. Altered ER-
MAM integrity can refer to any condition or state, including those that accompany AD,
where any structure or activity that is directly or indirectly related to a ER-MAM function has
been changed relative to a control or standard.

[00168] Without wishing to be bound by theory, pathogenic presenilin mutations
altered can be related to altered ER-MAM integrity. Alterations in ER-MAM function play a
role in the development of AD, for example by defects in mitochondrial distribution, and
mitochondrial dysfunction. Altered ER-MAM integrity can result from direct or indirect
effects of reduction, alteration or gain of function effects of mutations, in presenilin gene
products or related downstream mediator molecules and/or ER-MAM genes, gene products or
related downstream mediators, or from other known or unknown causes.

[00169] ER-MAM may contain gene products encoded by mitochondrial genes
situated in mitochondrial DNA (mtDNA) and by extramitochondrial genes (e.g., nuclear
genomes) not situated in the circular mitochondrial genome. Accordingly, mitochondrial and
extramitochondrial genes may interact directly, or indirectly via gene products and their
downstream intermediates, including metabolites, catabolites, substrates, precursors,
cofactors and the like. Alterations in ER-MAM integrity, for example altered APP
processing, amyloid toxicity, tau hyperphosphorylation, altered lipid and cholesterol
metabolism, altered glucose metabolism and mitochondrial dysfunction may therefore arise
as the result of defective mtDNA, defective extramitochondrial DNA, defective
mitochondrial or extramitochondrial gene products defective downstream intermediates or a
combination of these and other factors.

Indicators of Altered ER-MAM Integrity: Communication Between ER and Mitochondria

[00170] The communication between the ER and mitochondria in fibroblasts from
patients with FAD harboring pathogenic mutations in or FAD^{PS1} or FAD^{PS2} is increased
compared to controls. This increase in ER-MAM quantity also occurs in cells overexpressing
presenilin and in cells where presenilin is reduced by shRNA technology. Accordingly,
certain aspects of the invention are directed to methods for diagnosing Alzheimer's disease in
a subject, the method comprising comparing the communication between the ER and mitochondria in a biological sample to ER-MAM content of a control sample, wherein a increase in communication between the ER and mitochondria in the biological sample compared to the control indicates that the biological sample is from a subject having AD. One skilled in the art can determine the communication between the ER and mitochondria in a biological sample using assays for total protein or and/or total lipids in ER-MAM or total amount of ER-MAM resident proteins or ER-MAM resident lipids.

Further, the mitochondrial distribution is different in fibroblasts between age-matched controls and patients with FAD harboring pathogenic mutations in PS1 (FAD<sup>PS1</sup>): (1) Almost all the FAD<sup>PS1</sup> mitochondria are in the perinuclear region; (2) Fewer FAD<sup>PS1</sup> mitochondria are in the extremities of fibroblasts as compared to control; (3) FAD<sup>PS1</sup> mitochondria appear less elongated (e.g. less tubular) and more "punctate"; and (4) The communication between the ER and mitochondria in FAD<sup>PS1</sup> cells is significantly increased as compared to controls.

For Sporadic AD (SAD), there is also a difference in mitochondrial distribution. For SAD patients, there are three alleles of apolipoprotein E in humans: ApoE2, ApoE3, and ApoE4. People with at least one ApoE4 allele are at great risk for sporadic AD. ApoE4 is a MAM-localized protein. The mitochondrial distribution is: (1) Cells with E3/E3 have a normal MAM content; (2) Cells with E3/E4 have increased MAM, irrespective of whether or not the cells have a PS1 mutation; (3) Cells with PS1 mutation and E3/E3 genotype have normal amount of communication between the ER and mitochondria and normal mitochondrial distribution; (4) Cells with PS1 mutation and E3/E4 genotype have increased MAM and altered mitochondrial distribution; and (5) Similar results with brain tissue from PS1 patients: the communication between the ER and mitochondria in E3/E4 patients was increased compared to E3/E3.

Thus, without being bound by theory, the observation that there is a increased communication between the ER and mitochondria may help to explain the role of ApoE in the pathogenesis of AD, and may connect the familial and sporadic forms of the disease into one conceptual framework.

Indicators of ER-MAM Integrity: Protein Quantity in ER-MAM

Methods for determining ER-MAM-associated protein quantity can depend on the physicochemical properties of an ER-MAM-associated protein. In some
embodiments, determination of ER-MAM-associated protein quantity can involve quantitative determination of the level of a protein or polypeptide using routine methods in protein chemistry with which those having skill in the art. Depending on the nature and physicochemical properties of the ER-MAM-associated protein, determination of enzyme quantity can be by densitometric, mass spectrometric, spectrophotometric, fluorimetric, immunometric, chromatographic, electrochemical or any other means of quantitatively detecting a cellular component (See, e.g., Harlow and Lane, *Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory*, 1988; Weir, D. M., Handbook of Experimental Immunology, 1986, Blackwell Scientific, Boston). Methods for determining ER-MAM-associated protein quantity also include methods described that are useful for detecting products of enzyme catalytic activity, including those measuring enzyme quantity directly and those measuring a detectable label or reporter moiety.

[00175] The amount of an ER-MAM-associated protein, for example, can be determined in a gel pattern from a whole tissue, and in a gel pattern obtained using purified ER-MAM fraction. In the first pattern, the ER-MAM-associated protein can be a minor spot, in the latter, a major spot. The ratio of spot intensity for protein of a purified ER-MAM fraction can be referenced the ER-MAM-associated protein. The ratio between the ER-MAM-associated protein intensity on whole tissue gels and on the gels from isolated nuclei can be used as a multiplier to calculate the quantity of minor proteins in the whole tissue sample.

[00176] The proteins in a subcellular fraction can separated by a method that provides discrimination and resolution. For example, the proteins can be separated by known methods, such as chromatography, Immunoelectrophoresis, mass spectrometry or electrophoresis. The proteins can be separated in a liquid phase in combination with a solid phase. For example, a suitable separation method is two-dimensional (2-D) gel electrophoresis.

[00177] In one embodiment, isolated ER-MAM can also be assayed for the ratio of Aβ42:Aβ40 by Western blot or ELISA, wherein a greater ratio of Aβ42 to Aβ40 in isolated ER-MAM in a biological sample compared to the ratio of Aβ42 to Aβ40 in isolated ER-MAM in a control biological sample indicates that the subject has, or is at risk of having AD.

[00178] For example, assays can be performed in a Western blot format, wherein a preparation comprising proteins from a biological sample is submitted to gel electrophoresis,
transferred to a suitable membrane and allowed to react with an antibody specific for an ER-MAM-associated protein. The presence of the antibody on the membrane can then be detected using a suitable detection reagent, as is well known in the art and described herein.

[00179] For these and other useful affinity techniques, see, for example, Scopes, R.

[00180] In certain embodiments of the invention, an indicator of altered ER-MAM integrity including, for example, an ER-MAM-associated protein as provided herein, can be present in isolated form. Affinity techniques can be used to isolate an ER-MAM-associated protein and can include any method that exploits a specific binding interaction involving an ER-MAM-associated protein to effect a separation.

Indicators of Altered ER-MAM Function: Protein Activity

[00181] Certain aspects of the invention are directed to a method for diagnosing Alzheimer's disease in a subject comprising comparing measuring the activity of an ER-MAM-associated protein. In some embodiments of the invention, the activity of an ER-MAM-associated protein can be the indicator of altered ER-MAM integrity. In one embodiment, the indicator of altered ER-MAM integrity can refer to an indicator of altered ER-MAM integrity as provided herein, which is quantified in relation to activity of an ER-MAM-associated protein. For example, an indicator of altered ER-MAM integrity can be protein activity or enzymatic activity of an ER-MAM-associated protein determined on the basis of its level per unit ER-MAM-associated protein in a sample (e.g., ER-MAM-associated protein in the sample can be the non-enzyme indicator of altered ER-MAM integrity), but the invention need not be so limited.

[00182] In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample from an individual suspected of having Alzheimer's disease, (b) measuring the activity of non-presenilin an ER-MAM-associated protein in the biological sample of step (a), and (c) comparing the amount of activity of a non-presenilin ER-MAM-associated protein measured
in the biological sample of step (a) to the amount of activity of a non-presenilin ER-MAM-associated protein measured in a control biological sample wherein, an altered amount of activity of a non-presenilin ER-MAM-associated protein measured in the biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease.

[00183] As provided herein, the activity of proteins suitable for use as indicators or ER-MAM integrity include, but is are not limited to: Acyl-CoA:cholesterol acyltransferase (ACAT1); Acyl-CoA desaturase (stearoyl-CoA desaturase 1); Apolipoprotein E; Autocrine motility factor receptor 2 (GP78); β-galactoside a(2-3) sialyltransferase (SIAT4); β-galactoside a(2-6) sialyltransferase (SIAT1); β-1,4 N-acetylglactosaminyltransferase 1(SIAT2); P-1,4-galactosyltransferase 6 (lactosyl-ceramide synthase); Ceramide glucosyltransferase; Diacylglycerol O-acyltransferase; Fatty acid-CoA ligase, long-chain 1 (FACL1) (acyl-CoA synthetase 1); Fatty acid-CoA ligase, long-chain 4 (FACL4) (acyl-CoA synthetase 4); Fatty acid transport protein 4 (FATP4); Glucose-6-phosphatase; Glucose-regulated protein 78-kDa (BiP); Inositol 1,4,5-triphosphate receptor, type 3 (IP3R3); Microsomal triglyceride transfer protein large subunit; N-acetylglucosaminylphosphatidylinositol de-N-acetylasel; Opioid receptor, signal; Phosphatidylethanolamine N-methyltransferase 2 (PEMT); Phosphatidylinositol synthase 1 (PSS1); Phosphatidylinositol synthase 2 (PSS2); Phosphofurin acidic cluster sorting protein 2; Presenilin 1; Presenilin 2; Ryanodine Receptor type 1; Ryanodine Receptor type 2; Ryanodine Receptor type 3; Amyloid beta precursor protein; Basigin/CD147/EMMPRIN; Cytomegalus virus-encoded vMIA protein fr unsplinced exon I UL37 mRNA, N-term frag; Glucose-regulated protein 75-kDa (GRP75; Mortalin-2); and Membrane bound O-acyltransferase domain containing 2.


[00185] Products of enzyme catalytic activity can be detected by suitable methods that can depend on the quantity and physicochemical properties of the product. Thus, detection can be, for example by way of illustration and not limitation, by radiometric,
calorimetric, spectrophotometric, fluorimetric, immunometric or mass spectrometric procedures, or by other suitable means that will be readily apparent to a person having ordinary skill in the art.

[00186] In certain embodiments of the invention, detection of a product of enzyme catalytic activity can be accomplished directly, and in certain other embodiments detection of a product can be accomplished by introduction of a detectable reporter moiety or label into a substrate or reactant such as a marker enzyme, dye, radionuclide, luminescent group, fluorescent group or biotin, or the like. The amount of such a label that is present as unreacted substrate and/or as reaction product, following a reaction to assay enzyme catalytic activity, can then be determined using a method appropriate for the specific detectable reporter moiety or label. For radioactive groups, radionuclide decay monitoring, scintillation counting, scintillation proximity assays (SPA) or autoradiographic methods are appropriate.

[00187] For many proteins having enzymatic activity, including ER-MAM-associated proteins, quantitative criteria for enzyme catalytic activity are well established.

[00188] Methods for measure the activity of lipid biosynthetic enzymes are also known to those skilled in the art. For example, the activity of 3-hydroxy-3-methylglutaryl-CoA reductase can be measured (George et al, 1990). Acyl-CoA:cholesterol acyltransferase and diacylglycerol acyltransferase can be assayed in the same reaction mixture by a modification of the procedure of Heider et al. (1983) using [14C]oleoyl-CoA as substrate. Two of the products of the reaction, triacylglycerol and cholesteryl esters, can be separated by thin-layer chromatography in the solvent system hexane:ethyl acetate 9:1 (v/v). Phosphatidylserine synthase (base-exchange enzyme) can be assayed by methods known to those skilled in the art (Vance and Vance, 1988). CDP-choline-1,2-diacylglycerol cholinephosphotransferase and CDP-ethanolamine-1,2-diacylglycerol ethanolaminephosphotransferase activities can be measured by established procedures (Vance and Vance, 1988). PtdEtn N-methyltransferase activity can be assayed using exogenously added phosphatidyl(1-monoo)ethanolamine as substrate (Vance and Vance, 1988). In some embodiments of any enzymatic assay described herein, Triton X-100 can be omitted from the protocol.

Indicators of Altered ER-MAM Function: ATP Biosynthesis

[00189] In one embodiment of the invention, a mitochondrial protein activity can be the indicator of altered ER-MAM integrity. The enzyme may be a mitochondrial enzyme,
which may further be an ETC enzyme or a Krebs cycle enzyme. In other embodiments, the indicator of ER-MAM integrity is any ATP biosynthesis factor. Accordingly, the indicator of ER-MAM integrity, may comprise a measure of the function of an electron transport chain (ETC) enzyme, which refers to any mitochondrial molecular component that is a mitochondrial enzyme component of the mitochondrial electron transport chain (ETC) complex associated with the inner mitochondrial membrane and mitochondrial matrix. An ETC enzyme may include any of the multiple ETC subunit polypeptides encoded by mitochondrial and nuclear genes. The ETC can comprise complex I (NADH:ubiquinone reductase), complex II (succinate dehydrogenase), complex III (ubiquinone: cytochrome c oxidoreductase), complex IV (cytochrome c oxidase) and complex V (mitochondrial ATP synthetase), where each complex includes multiple polypeptides and cofactors (for review see, e.g., Walker et al., 1995 Meths).

Indicators of Altered ER-MAM Function: Phosphatidylethanolamme Synthesis

[00190] The ER-MAM is a locus of phospholipid synthesis. Phosphatidylserine (PS) is transported from the MAM to mitochondria, where it is decarboxylated to phosphatidylethanolamme (PE). The PE is then re-transported back to the ER-MAM, where it is methylated to phosphatidylcholine (PC). When ER-MAM is increased, the rate of transport of PS from the MAM to the mitochondria is increased, and the production of PE inside of mitochondria is also increased.

[00191] For example, by way of illustration and not limitation, an ER-MAM-associated protein that is an enzyme can refer to a trans-membrane transporter molecule that, through its enzyme catalytic activity, facilitates the movement of metabolites between cellular compartments. For example, such metabolites can include, but are not limited to phosphatidylserine, phosphatidylethanolamme or other cellular components involved in phosphatidylcholine synthesis, such as gene products and their downstream intermediates, including metabolites, catabolites, substrates, precursors, cofactors and the like.

[00192] In one aspect, the invention provides a method for diagnosing Alzheimer's disease in a subject, the method comprising: (a) obtaining a biological sample from an individual suspected of having Alzheimer's disease, (b) measuring the rate of conversion of phosphatidylserine to phosphatidylethanolamme in the biological sample of step (a), and (c) comparing the rate of conversion of phosphatidylserine to phosphatidylethanolamme measured in the biological sample of step (a) to the rate of conversion of phosphatidylserine
to phosphatidylethanolamme measured in a control biological sample wherein, an increased rate of conversion of phosphatidylserine to phosphatidylethanolamme measured in the biological sample of step (a) compared to the control biological sample indicates that the subject has Alzheimer's disease.

The rate of conversion of phosphatidylserine to phosphatidylethanolamme can be measured, for example, by adding \(^3\)H-Ser to cells and measuring the amount of \([\text{^3}H]\text{PE}\) (and \([\text{^3}H]\text{PS}\)) produced as a function of time (Achleitner et al. (1995) J. Biol. Chem. 270, 29836). In a diagnostic setting, \(^3\)H-Ser incorporation in any easily available cell from AD patients can be measured and compared to controls.

Cholesterol and phospholipids (e.g. PE, PS, and PC) are selectively reduced in a mouse AD "double-transgenic" (i.e. mutations in both APP and PS1) model (Yao et al. (2008) Neurochem. Res. in press). When ER-MAM is increased, the steady-state levels of PE in cellular membranes, including the plasma membrane, will be increased.

In some embodiments of the invention, a change in the amount of PE in an AD cell can be used as a diagnostic marker. Cinnamycin (also called Ro 09-0198) is a tetracyclic peptide antibiotic that can be used to monitor transbilayer movement of PE in biological membranes because it binds specifically to PE. When bound, cinnamycin forms a 1:1 complex with PE (Choung et al. (1988) Biochem. Biophys. Acta 940:171). Cinnamycin has been used to identify mutants defective in PS transport through the MAM (Emoto et al. (1999) PNAS 96:12400). Pore formation and hemolysis occurs upon binding of cinnamycin to PE containing membranes and thus AD cells (as a result of greater amount of PE in cell membranes) will be more susceptible to cytolysis and cinnamycin-induced killing at lower concentrations of cinnamycin as compared to control cells.

One of skill in the art will appreciate that duramycin can also be used to detect phosphatidylethanolamme and can be used interchangeably with cinnamycin according to the methods described herein.

One of skill in the will also understand that papuamide B (derived from a sea sponge found in Papua, New Guinea) binds specifically to phosphatidylserine (PtdSer) (Parsons et al., 2006) and can be used in a manner analogous to cinnamycin/duramycin to detect PtdSer on the cell surface in accordance with the methods disclosed herein (Chen et al., 2006). One of skill in the art would readily be capable of determining whether other compounds in the class of papuamide B, including, but not limited to papuamides A-D;
(Andjelic et al., 2008), callipeltin A, neamphamide A, and mirabimides A-D (Xie et al., 2008; Oku et al., 2005) are can be readily adopted for use with the methods described herein. One of skill in the art will also appreciate that Annexin V and Lactadherin have long been used to detect PtdSer on the cell surface (Metkar et al., 2011) and thus could be adapted for use with the methods described herein.

Indicators of Altered ER-MAM Integrity: Presenilin Localization

[00198] As described herein, PS1 and PS2 are enriched in ER-MAM. In certain embodiments, the localization of PS1 or PS2 to perinuclear regions is an indicator of altered ER-MAM integrity

[00199] Methods for measuring the amount of in ER-MAM are known to those skilled in the art. For example, total presenilin protein in a ER-MAM can be determined by subcellular fractionation and Western blotting. Total presenilin protein in a ER-MAM can also be determined by immunohistochemistry by comparing the amount of co-localization between presenilin and a known ER-MAM resident protein, for example PEMT.

Indicators of Altered ER-MAM Integrity: Mitochondrial Localization or Morphology

[00200] Mitochondria are organelles found in most mammalian cells. They are the location of many "housekeeping" functions, foremost among them the production of energy in the form of ATP via the respiratory chain/oxidative phosphorylation system. This aspect of mitochondrial function is unique, because the production of oxidative energy is a joint venture between the mitochondrion and the nucleus: genes from both organelles are required. Mitochondria are plastic, with shapes that vary from small spheres (~1 µm in diameter) to highly elongated tubular structures. In normal cells, they can exist as linear "strings" or as highly branched, reticular structures.

[00201] All but 13 of the ~1,000 proteins present in mitochondria are encoded by nuclear DNA (nDNA). They are synthesized in the cytoplasm and are targeted to mitochondria via mitochondrial targeting signals (MTS's) that direct the polypeptides not only to mitochondria, but also to the proper compartment within the organelle (the outer membrane (MOM), the intermembrane space (IMS), the inner membrane (MIM), and the matrix). The MTS's that target polypeptides to the inner membrane and matrix can have N-terminal presequences that are cleaved following importation. However, much less is known regarding the MTS's of polypeptides that are targeted to the MOM or to the IMS: some are C-
terminal and some are "internal," located within the "business end" of the protein. These MTS's are not cleaved off following importation.

[00202] The results described herein show that the distribution of mitochondria in fibroblasts from patients with FAD harboring pathogenic mutations in presenilin is different from the distribution of mitochondria in age-matched normal control fibroblasts. Most mitochondria in FAD$^{PS1}$ or FAD$^{PS2}$ cells are in the perinuclear region, with fewer mitochondria in the "extremities" of the fibroblasts as compared to control cells. In addition, the mitochondria appear less elongated (e.g. less tubular) and more "punctate." In certain embodiments, the localization of PS1 or PS2 is a indicator of altered ER-MAM integrity.

[00203] Methods for quantifying mitochondrial localization or mitochondrial morphology are known in the art, and can include, for example, quantitative staining of a representative biological sample. By way of example, quantitative staining of mitochondrial can be performed using organelle-selective probes or dyes, including but not limited to mitochondrial selective reagents such as fluorescent dyes that bind to mitochondrial components (e.g., nonylacridine orange, MitoTrackers™) or potentiometric dyes that accumulate in mitochondria as a function of mitochondrial inner membrane electrochemical potential (see, e.g., Haugland, 1996 Handbook of Fluorescent Probes and Research Chemicals—Sixth Ed., Molecular Probes, Eugene, Oreg.)

[00204] Mitochondrial mass, volume and/or number can be quantified by morphometric analysis (e.g., Cruz-Orive et al., 1990 Am. J. Physiol. 258:L148; Schwerzmann et al., 1986 J. Cell Biol. 102:97). These or any other means known in the art for quantifying mitochondrial localization or mitochondrial morphology in a sample are within the scope of the invention. Calculations of mitochondrial density can be performed, can include, but are not limited to the use of such quantitative determinations. In some embodiments, mitochondrial localization or mitochondrial morphology can be determined using well known procedures. For example, a person having ordinary skill in the art can readily prepare one or more cells from a biological sample using established techniques, and therefrom determine mitochondrial localization or mitochondrial morphology protein content using any of a number of visualization methodologies well known in the art.

[00205] Methods for determining the ratio perinuclear mitochondria to non-perinuclear mitochondria and the ratio of punctate mitochondria to non-punctate mitochondria in a cell are known to those skilled in the art. For example the amount of
perinuclear mitochondria is a cell can be determined by confocal microscopy. Confocal imaging z sections can be projected into a single image. An area between the nucleus and the cell periphery, as determined by microtubule staining, can be outlined, and the midpoint between the nucleus and the farthest point at the cell periphery can be determined. Using the midpoint, the outlined area is then divided into two parts: regions proximal (A) and distal (B) to the nucleus. Mean grayness values of the MitoTracker stain are recorded for the proximal and distal parts. For quantification of mitochondria in the outer edges of a cell, the grayness value for the distal part can be divided by the grayness value for the total area (proximal + distal). Grayness value for the total area = ([GraynessA x AreaA] + [GraynessB x AreaB])/(AreaA + AreaB).


**Indicators of Altered ER-MAM Integrity: Mitochondrial movement**

[00207] Mitochondria can fuse and divide, and are also mobile. In mammalian cells they move predominantly along microtubules. This movement, which requires a membrane potential, can be important in neurons, where mitochondria travel from the cell body to the cell’s extremities at the ends of axons and dendrites, in order to provide energy for pre-synaptic transmission and for post-synaptic uptake of critical small molecules (e.g. neurotransmitters). Mitochondria attach to microtubules via kinesins and dyneins (Zhang et al, 2004). At least three mitochondrial-binding kinesins have been identified: KIFIB, KIF5B, and KLC3. The binding of kinesins is regulated by phosphorylation by glycogen synthase
kinase 3β (GSK3β). The kinetics of mitochondrial attachment to kinesin is also mediated by the microtubule associated protein tau (Trinczek et al., 1999), which is also a target of GSK3β (Tatebayashi et al., 2004). Tau affects the frequency of attachment and detachment of mitochondria to the microtubule tracks (Trinczek et al., 1999). In S. cerevisiae mitochondria move along actin cables, but in S. pombe and mammalian cells they move mainly along microtubules. This movement is important in neurons, where mitochondria travel from the cell body to the cell’s extremities at the ends of axons and dendrites, in order to provide energy for pre synaptic transmission and for post-synaptic uptake of critical small molecules (e.g. neurotransmitters). Without mitochondrial movement, metazoan life would not exist.

[00208] The dynamics of mitochondrial fusion and fission can be examined using mitochondrially-targeted photo-activatable fluorescent probes ("mitoDendra") and live-cell imaging of neuronal cells to examine the effects of presenilin mutations in mitochondrial distribution. Mitochondrial maldistribution AD can occur as a result of defects in anterograde and retrograde axonal transport of mitochondria. Mitochondrial maldistribution AD can also occur as a consequence of retention and/or accumulation of mitochondria the extremities of cells. For example, defects in anterograde and retrograde axonal transport of mitochondria, on retention and accumulation of mitochondria in nerve terminals, and on the dynamics of mitochondrial fusion and fission in AD can be performed in primary neuronal cells derived from normal, FAD<sup>Ps1</sup> and FAD<sup>Ps2</sup> mice.

[00209] In one embodiment, mitochondrial movement is measured using a mitochondrially targeted Mitotracker dye and live-cell imaging. In another embodiment, mitochondrial movement is measured using a mitochondrially targeted photo-activatable GFP ("mitoDendra") and live-cell imaging. Dendra is a monomeric variant of GFP ("dendGFP") that changes from green to red fluorescent states when photoactivated by 488-nm light. Dendra is stable at 37°C and photoconversion of the photoactivatable GFP from green to red is irreversible and photostable (Gurskaya et al., 2006) Engineering of a monomeric green-to-red photo-activatable fluorescent protein induced by blue light. Nat. Biotechnol. 24:461-465). For example, individual mitochondria can be converted to red fluorescence to track movement in the cell body, to determine whether they appear in a specified distance downstream in an axon, and how long it took to get there.

[00210] Because the mitochondrial mislocalization phenotype can be due to (1) a reduced ability of mitochondria to move efficiently along microtubules, or (2) a reduced
ability of mitochondria to attach to microtubules (or some combination of the two), mitochondria can be visualized in living cells by colocalizing red mito-Dendra with TubulinTracker Green (a bi-acetylated version of Oregon Green 488 paclitaxel; Molecular Probes T34075) to determine if they are attached to microtubules.

[00211] Mitochondrial movement can be examined along with interaction with microtubules and microtubule-based motors in presenilin-ablated neurons focusing on the relationship between presenilin, GSK3β, tau, and kinesins. Presenilin-associated defects in mitochondrial distribution can also be examined to determine if they affect energy mobilization, and the extent to which mitochondrial distribution defects contribute to neuronal dysfunction in presenilin-ablated neurons.

[00212] Alterations in mitochondrial function, for example impaired electron transport activity, defective oxidative phosphorylation or increased free radical production, can also arise as the result of defective mitochondria movement or localization. In one embodiment of the invention, a mitochondrial protein activity can be the indicator of altered ER-MAM integrity. The enzyme can be a mitochondrial enzyme, which can further be an electron transport chain enzyme or a Krebs cycle enzyme, or other enzymes or cellular components related to ATP production.

Indicators of Altered ER-MAM Integrity: Free Radical Production

[00213] In certain embodiments of the invention, free radical production in a biological sample can be detected as an indicator of altered ER-MAM integrity. Without wishing to be bound by theory, increased communication between ER and mitochondria can result in elevated reactive oxygen species (ROS).

[00214] Accordingly, an indicator of altered ER-MAM integrity can be a free radical species present in a biological sample (e.g. reactive oxygen species). Methods of detecting free radicals are known in the art, and such methods include, but are not limited to fluorescent and/or chemiluminescent indicators (see Handbook of Methods for Oxygen Radical Research, 1985 CRC Press, Boca Raton, Fla; Molecular Probes On-line Handbook of Fluorescent Probes and Research Chemicals, at http://www.probes.com/handbook/toc.html). Free radical mediated damage to mitochondria can also result in collapse of the electrochemical potential maintained by the inner mitochondrial membrane. Methods for detecting changes in the inner mitochondrial membrane potential are described herein and in U.S. patent application Ser. No. 09/161,172.
Although mitochondria are a primary source of free radicals in biological systems (see, e.g., Murphy et al., 1998 in *Mitochondria and Free Radicals in Neurodegenerative Diseases*, Beal, Howell and Bodis-Wollner, Eds., Wiley-Liss, New York, pp. 159-186 and references cited therein), the invention should not be so limited and free radical production can be an indicator of altered ER-MAM integrity regardless of the subcellular source site.

In one embodiment, reactive oxygen species (e.g. superoxide, hydrogen peroxide, singlet oxygen, and peroxynitrite) can be measured by using Mitosox Red (Molecular Probes). Mitosox Red is live-cell permeant and is selectively targeted to mitochondria. Once inside the mitochondria, the reagent is oxidized by superoxide and binds to nucleic acids, resulting in a red fluorescence. Increased Mitosox staining occurs in presenilin mutant cells compared to control cells (see Example 1).

In another embodiment, reactive oxygen species can be measured with 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-FLDCFDA) in a "Image-iT Live" assay (Molecular Probes). Carboxy-FLDCFDA is a fluorogenic marker for reactive oxygen species and is deacetylated by nonspecific intracellular esterases. In the presence of reactive oxygen species, the reduced fluorescein compound is oxidized and emits bright green fluorescence. Methods for detecting a free radical that may be useful as an indicator of altered ER-MAM integrity are known in the art and will depend on the particular radical.

**Indicators of ER-MAM Integrity: Alterations of Calcium Levels**

Certain aspects of the present invention, as it relates to the correlation of Alzheimer's disease with an indicator of altered ER-MAM integrity, involve monitoring intracellular calcium homeostasis and/or cellular responses to perturbations of this homeostasis, including physiological and pathophysiological calcium regulation.

As described herein, PS1 is a regulator of Ca2+ storage in the ER and PS1 exerts an effect on ER-mitochondrial Ca2+ transfer, sensitizing mitochondria to permeabilization in FAD^PS1 cells, leading to cell injury. Thus, according to certain embodiments of the present invention, release of ER-stored Ca2+ can potentiate influx of cytosolic free calcium into the mitochondria, as can occur under certain physiological conditions including those encountered by cells of a subject having increased communication between ER and mitochondria. Detection of such changes in calcium concentrations can be accomplished by a variety of means (see, e.g., Ernster et al., *Cell Biol.* 91:227 (1981);


**[00221]** As described herein, mutations in presenilins (or loss of presenilin function) can cause variation of ER, mitochondrial or cytosolic calcium levels from standard physiological ranges. In Alzheimer disease cells, mitochondrial calcium levels can be increased about 50% above the values in normal cells, and cytosolic Ca\(^{2+}\) can be increased by about 25% (i.e. from around 175 nM in normal cells to around 220 nM in AD cells after stimulation by exogenously-added ATP).

**Indicators of ER-MAM Integrity: Protein Interactions**

**[00222]** Methods for determining ER-MAM-associated protein interactions can depend on the physicochemical properties of an ER-MAM-associated protein. In some embodiments, determination of ER-MAM-associated protein interactions can involve quantitative determination of the level of a protein or polypeptide interaction using routine methods known in the art (See, e.g., Harlow and Lane, *Antibodies: A Laboratory Manual*,

[00223] In some embodiments of the invention, the association between one of more ER-MAM-associated proteins can be the indicator of altered ER-MAM integrity and can be used to determine whether a subject has, or is predisposed to having Alzheimer's disease.

[00224] As provided herein, associating ER-MAM-associated proteins can include, but are not limited to Acyl-CoA:cholesterol acyltransferase (ACAT1); Acyl-CoA desaturase (stearoyl-CoA desaturase 1); Apolipoprotein E; Autocrine motility factor receptor 2 (GP78); β-galactoside a(2-3) sialyltransferase (SIAT4); β-galactoside a(2-6) sialyltransferase (SIAT1); β-1,4 N-acetylgalactosaminyltransferase 1(SIAT2); P-1,4-galactosyltransferase 6 (lactosyl-ceramide synthase); Ceramide glucosyltransferase; Diacylglycerol O-acyltransferase; Fatty acid-CoA ligase, long-chain 1 (FACL1) (acyl-CoA synthetase 1); Fatty acid-CoA ligase, long-chain 4 (FACL4) (acyl-CoA synthetase 4); Fatty acid transport protein 4 (FATP4); Glucose-6-phosphatase; Glucose-regulated protein 78-kDa (BiP); Inositol 1,4,5-triphosphate receptor, type 3 (IP3R3); Microsomal triglyceride transfer protein large subunit; N-acetylgalactosaminyl-phosphatidylinositol de-N-acetylase; Opioid receptor, signal; Phosphatidylethanolamine N-methyltransferase 2 (PEMT); Phosphatidylycerine synthase 1 (PSS1); Phosphatidylycerine synthase 2 (PSS2); Phosphofurin acidic cluster sorting protein 2; Presenilin 1; Presenilin 2; Ryanodine Receptor type 1; Ryanodine Receptor type 2; Ryanodine Receptor type 3; Amyloid beta precursor protein; Basigin/CD147/EMMPRIN; Cytomegalus virus-encoded vMIA protein fr unsplced exon 1UL37 mRNA, N-term frag; Glucose-regulated protein 75-kDa (GRP75; Mortalin-2); and Membrane bound O-acyltransferase domain containing 2.

[00225] In one embodiment, an indicator of ER-MAM integrity is a modulation of the amount or character of a presenilin containing complex. The protein complexes and component proteins can be obtained by methods well known in the art for protein purification and recombinant protein expression. For example, the presenilin interaction partners can be isolated by immunoprecipitation from whole cell lysates or from purified cell fractions (e.g. ER-MAM cell fractions). In another embodiment, an indicator of ER-MAM integrity is an increase in the association of ER-MAM-associated proteins in a test biological sample (e.g. Diacylglycerol-O-acyltransferase 2 (DGAT2) and stearoyl-CoA desaturase 1 (SCD1)) to the association of ER-MAM-associated proteins in a control biological sample.
[00226] For recombinant expression of one or more of the proteins, the nucleic acid containing all or a portion of the nucleotide sequence encoding the protein can be inserted into an appropriate expression vector, i.e., a vector that contains the necessary elements for the transcription and translation of the inserted protein coding sequence. The necessary transcriptional and translational signals can also be supplied by the native promoter of the component protein gene, and/or flanking regions.

[00227] Assays for detecting, isolating and characterizing protein complexes are well known in the art (e.g., immunoassays, activity assays, mass-spectrometry, .. etc.) and can be used to determine whether one or more presenilin interaction partners are present at either increased or decreased levels, or are absent, in samples from patients suffering from AD, or having a predisposition to develop AD, as compared to the levels in samples from subjects not having AD, or having a predisposition to develop AD. Additionally, these assays can be used to determine whether the ratio of the complex to the un-complexed components in a presenilin containing protein complex, is increased or decreased in samples from patients suffering from AD, or having a predisposition to develop AD, as compared to the ratio in samples from subjects not having AD, or not having a predisposition to develop AD.

[00228] In the event that levels of one or more protein complexes (i.e., presenilin containing protein complexes) are determined to be increased in patients suffering from AD, or having a predisposition to develop AD, then the AD, or predisposition for AD, can be diagnosed, have prognosis defined for, be screened for, or be monitored by detecting increased levels of the one or more protein complexes, increased levels of the mRNA that encodes one or more members of the one or more protein complexes, or by detecting increased complex functional activity.

[00229] In the event that levels of one or more protein complexes (i.e., presenilin containing protein complexes) are determined to be altered in patients suffering from AD, or having a predisposition to develop AD, then the AD, or predisposition for AD, can be diagnosed, have prognosis defined for, be screened for, or be monitored by detecting altered levels of the one or more protein complexes, increased levels of the mRNA that encodes one or more members of the one or more protein complexes, or by detecting increased complex functional activity.

[00230] Accordingly, in one embodiment of the invention, AD involving aberrant compositions of presenilin containing protein complexes can be diagnosed, or their suspected
presence can be screened for, or a predisposition to develop such disorders can be detected,
by detecting the component proteins of one or more complexes from a whole cell lysate or
from a subcellular fraction of a cellular lysate (e.g. an ER-MAM fraction).

[00231] Methods for screening for a molecule that binds a presenilin protein
complex can be performed using cell-free and cell-based methods known in the art (e.g. in
vitro methods, in vivo methods or ex vivo methods). For example, an isolated PS1 protein
complex can be employed, or a cell can be contacted with the candidate molecule and the
complex can be isolated from such contacted cells and the isolated complex can be assayed
for activity or component composition.

[00232] Methods for screening can involve labeling the component proteins of the
complex with, for example, radioligands, fluorescent ligands or enzyme ligands. Presenilin
protein complexes can be isolated by any technique known in the art, including but not
restricted to, co-immunoprecipitation, immunoaffinity chromatography, size exclusion
chromatography, and gradient density centrifugation.

[00233] Suitable binding conditions are, for example, but not by way of limitation,
in an aqueous salt solution of 10-250 mM NaCl, 5-50 mM Tris-HCl, pH 5-8, and a detergent.
Suitable detergents can include, but are not limited to non-ionic detergents (for example, NP-
40) or other detergents that improves specificity of interaction. One skilled in the art will
readily be able to determine a suitable detergent and a suitable concentration for the
detergent. Metal chelators and/or divalent cations can be added to improve binding and/or
reduce proteolysis. Complexes can be assayed using routine protein binding assays to
determine optimal binding conditions for reproducible binding.

[00234] Binding species can also be covalently or non-covalently immobilized on a
substrate using any method well known in the art, for example, but not limited to the method
of Kadonaga and Tjian, 1986, Proc. Natl. Acad. Sci. USA 83:5889-5893, i.e., linkage to a
cyanogen-bromide derivatized substrate such as CNBr-Sepharose 4B (Pharmacia). Non-
covalent attachment of proteins to a substrate include, but are not limited to, attachment of a
protein to a charged surface, binding with specific antibodies and binding to a third unrelated
interacting protein.

[00235] Proteins of the complex can be cross-linked to enhance the stability of the
complex. Different methods to cross-link proteins are well known in the art. As is apparent to
a person skilled in the art, the optimal rate of cross-linking need to be determined on a case
by case basis. This can be achieved by methods well known in the art, some of which are exemplary described herein.

Indicators of ER-MAM Integrity as Measured by Fluorescence Resonance Energy Transfer

[00236] One indicator of ER-MAM integrity suitable for the purposes described herein is based on the known interaction between two ER-MAM-associated proteins - diacylglycerol-O-acyltransferase 2 (DGAT2) and stearoyl-CoA desaturase 1 (SCD1). These proteins form a dimeric complex in ER-MAM (Man et al. (2006) J. Lipid Res. 47:1928). When yellow fluorescent protein (CFP) is fused to DGAT2 (DGAT2-YFP) and cyan fluorescent protein (YFP) is fused to SCD1 (SCD1-YFP), illumination with light of the appropriate wavelength results in energy transfer from the YFP to the CFP (i.e. fluorescence resonant energy transfer) to yielding a visible signal. Fluorescence resonant energy transfer (FRET) occurs when the two proteins are within a few nanometers of one another. If the two polypeptides are separated from each other by even a few tens of nanometers, FRET does not occur.

[00237] In one embodiment, determining whether protein associations increase or are altered can be performed by, for example, (1) transfecting the cells obtained from the subject and the control cells with one or more expression vectors that express a DGAT2-CFP fusion protein and an SCD1-YFP fusion protein (or other FRET proteins); (2) illuminating the transfected cells with an appropriate wavelength of light to excite the YFP; and (3) comparing the fluorescent signal levels emitted from CFP in the transfected cells from the subject and the control.

[00238] Without wishing to be bound by theory, normal cells will have a weak FRET signal ER-MAM membrane structure. Conversely, when ER-MAM is increased in AD, ER-MAM structure is altered and associating ER-MAM proteins will traverse the membrane laterally in an altered manner. This will result in a increased FRET signal. This increase in FRET can be exploited as a tool for diagnosis of AD and as a tool for identifying compounds useful for the treatment or prevention of AD. For example, fibroblasts (or other cells) from AD patients can be transfected with DGAT2-CFP and SCD1-YFP and the FRET can be assayed. Increased signal is indicative of compromised ER-MAM, due to AD (or to any other pathology that affects ER-MAM integrity). Similarly, compounds can be screened for an ability to decrease a FRET signal in AD cells. For example, FADPS1 or FADPS2 cells can be transfected stably with a bicistronic vector containing DGAT2-CFP and SCD1-YFP,
but owing to the ER-MAM defect they will have low FRET. These cells can be treated with a library of compounds to identify compounds that reduce FRET signals.

[00239] In one embodiment, ER-MAM integrity can be determined by measuring FRET between yellow fluorescent protein (YFP) fused to DGAT2 (DGAT2-CFP) and cyan fluorescent protein (CFP) fused to SCD1 (SCD1-YFP) upon illumination with light of the appropriate wavelength and energy transferred from the YFP to the CFP (i.e. fluorescence resonant energy transfer (FRET) to yield a signal). In another embodiment, the donor fluorophore and acceptor are selected so that the donor fluorophore and acceptor exhibit resonance energy transfer when the donor fluorophore is excited. A fluorescence resonance energy transfer (FRET) pair comprises a donor fluorophore and an acceptor where the overlap between the emissions spectrum of the donor fluorophore and the absorbance spectrum of the acceptor is sufficient to enable FRET.

[00240] In another embodiment, ER-MAM integrity can be determined by using "dark FRET" by measuring energy transfer between an ER-MAM-associated protein (e.g. DGAT2) fused to a fluorescent donor and an ER-MAM (e.g. SCD1) protein fused to a non-fluorescent chromoprotein (Ganesan et al, Proc Natl Acad Sci USA. 2006 March 14; 103(1): 4089-4094). Suitable combinations of donor fluorophores and acceptor non-fluorescent chromoproteins, include, but are not limited to EYFP and REACh (Resonance Energy Accepting Chromoprotein) (Ganesan et al, Proc Natl Acad Sci USA. 2006 March 14; 103(1): 4089-4094). A non-fluorescent chromoprotein can be any fluorescent protein (or variant thereof) that retains its absorption properties and can act as a quencher for the donor fluorescence. FRET with a non-fluorescent chromoprotein can be visualized by changes in the donor emission: its reduced lifetime by fluorescence lifetime imaging, quenched emission in relation to a reference fluorophore, and delayed photobleaching kinetics.

[00241] ER-MAM-associated proteins fused to a fluorescent proteins (or non-fluorescent chromoproteins) can be readily generated by methods known in the art. Such fluorescent fusion proteins (or non-fluorescent chromoproteins) can be used to detect protein interaction by several methods, including but not limited to immunoprecipitation and fluorescence resonance energy transfer (FRET). A fluorescent protein (or non-fluorescent chromoprotein) can be specifically linked to the amino- or carboxyl-terminus of an ER-MAM-associated protein sequence using well known chemical methods, see, e.g., Chemical Approaches to Protein Engineering, in Protein Engineering: A Practical Approach (Eds. Rees et al, Oxford University Press, 1992). A fluorescent protein (or non-fluorescent
chromoprotein) can also be specifically inserted in-frame within an ER-MAM-associated protein using well-known chemical methods.

[00242] The ER-MAM fluorescent-fusion proteins (or non-fluorescent chromoproteins) disclosed in the present specification include, in part, donor fluorophore. As used herein, the term "fluorophore" is synonymous with the term "fluorochrome" or "fluorescent molecule." As used herein, the term "donor fluorophore" means a molecule that, when irradiated with light of a certain wavelength, emits light of a different wavelength, also denoted as fluorescence. Thus, a donor fluorophore can be a fluorescent molecule.

[00243] The ER-MAM fluorescent fusion proteins disclosed in the present specification include, in part, acceptor. As used herein, the term "acceptor" means a molecule that can absorb energy from a donor fluorophore and is a term that encompasses fluorescent molecules as well as non-fluorescent molecules. As used herein, the term "acceptor fluorophore" means an acceptor comprising a fluorescent molecule or any non-fluorescent chromoprotein. Any fluorescent molecules can serve as a donor fluorophore or an acceptor fluorophore, including, without limitation, a fluorescent protein, a fluorophore binding protein and a fluorescent dye.

[00244] A donor fluorophore or an acceptor fluorophore disclosed in the present specification can be, in part, a fluorescent protein. As used herein, the term "fluorescent protein" means a peptide which absorbs light energy of a certain wavelength and emits light energy of a different wavelength and encompasses those which emit in a variety of spectra, including violet, blue, cyan, green, yellow, orange and red. Fluorescent proteins derived from any of a variety of species can be useful in aspects of the present invention including, but not limited to, Aequorea fluorescent proteins, Anemonia fluorescent proteins, Anthozoa fluorescent proteins, Discosoma fluorescent proteins, Entacmeae fluorescent proteins, Heteractis fluorescent proteins, Montastrea fluorescent proteins, Renilla fluorescent proteins, Zoanthus fluorescent proteins, and fluorescent proteins from other organisms. Fluorescent proteins useful in the invention encompass, without limitation, wild type fluorescent proteins, naturally occurring variants, and genetically engineered variants, produced, e.g., by random mutagenesis or rational designed, and active peptide fragments derived from an organism.

[00245] Fluorescent proteins (or non-fluorescent chromoproteins) useful in aspects of the invention include, e.g., those which have been genetically engineered for superior performance such as, without limitation, altered excitation or emission wavelengths;
enhanced brightness, pH resistance, stability or speed of fluorescent protein formation; photoactivation; or reduced oligomerization or photobleaching. see, e.g., Brendan P. Cormack et al, FACS-optimized Mutants of the Green Fluorescent Protein (GFP), U.S. Pat. No. 5,804,387 (Sep. 8, 1998); Roger Y. Tsien & Roger Heim, Modified Green Fluorescent Proteins, U.S. Pat. No. 6,800,733 (Oct. 5, 2004); Roger Y. Tsien et al, Long Wavelength Engineered Fluorescent Proteins, U.S. Pat. No. 6,780,975 (Aug. 24, 2004); and Roger Y. Tsien et al., Fluorescent Protein Sensors For Measuring the pH of a Biological Sample, U.S. Pat. No. 6,627,449 (Sep. 30, 2003).

[00246] A fluorescent protein (or non-fluorescent chromoprotein) can be engineered for improved protein expression by converting wild type codons to other codons more efficiently utilized in the cells which serve to express the ER-MAM-associated protein, see, e.g., Brian Seed and Jurgen Haas, High Level Expression of Proteins, U.S. Pat. No. 5,795,737 (Aug. 18, 1998). A fluorescent protein (or non-fluorescent chromoprotein) can be operably-linked to an ER-MAM-associated protein to create a fusion protein using standard molecular genetic techniques. In one aspect, the ER-MAM-associated protein can be any of Acyl-CoA:cholesterol acyltransferase (ACAT1); Acyl-CoA desaturase (stearoyl-CoA desaturase 1); Apolipoprotein E; Autocrine motility factor receptor 2 (GP78); β-galactoside a(2-3) sialyltransferase (SIAT4); β-galactoside a(2-6) sialyltransferase (SIAT1); β-1,4 N-acetylgalactosaminytransferase 1(SIAT2); P-1,4-galactosyltransferase 6 (lactosyl-ceramide synthase); Ceramide glucosyltransferase; Diacylglycerol O-acyltransferase; Fatty acid-CoA ligase, long-chain 1 (FACL1) (acyl-CoA synthetase 1); Fatty acid-CoA ligase, long-chain 4 (FACL4) (acyl-CoA synthetase 4); Fatty acid transport protein 4 (FATP4); Glucose-6-phosphatase; Glucose-regulated protein 78-kDa (BiP); Inositol 1,4,5-triphosphate receptor, type 3 (IP3R3); Microsomal triglyceride transfer protein large subunit; N-acetylglycosaminyl-phosphatidylinositol de-N-acetylas; Opioid receptor, signal; Phosphatidylethanolamine N-methyltransferase 2 (PEMT); Phosphatidylinerine synthase 1 (PSS1); Phosphatidylinerine synthase 2 (PSS2); Phosphohorul acidic cluster sorting protein 2; Presenilin 1; Presenilin 2; Ryanodine Receptor type 1; Ryanodine Receptor type 2; Ryanodine Receptor type 3; Amyloid beta precursor protein; Basigin/CD147/EMMPRIN; Cytomegalus virus-encoded vMIA protein fr unspliced exon 1UL37 mRNA, N-term frag; Glucose-regulated protein 75-kDa (GRP75; Mortalin-2); and Membrane bound O-acyltransferase domain containing 2.

[00247] Any of a variety of fluorescently active protein fragments can be useful in aspects of the present invention with the proviso that these active fragments retain the ability
to emit light energy in a range suitable for the proper operation of aspects of the present invention, such as, e.g. about 420-460 nm for blue emitting fluorescent proteins, about 460-500 nm for cyan emitting fluorescent proteins, about 500-520 nm for green emitting fluorescent proteins, about 520-550 nm for yellow emitting fluorescent proteins and about 550-740 nm for red emitting fluorescent proteins (Table 3).

Table 3. Excitation and Emission Maxima of Exemplary Fluorescent Proteins

<table>
<thead>
<tr>
<th>Fluorescent proteins</th>
<th>Excitation Maxima (nm)</th>
<th>Emission Maxima (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBFP</td>
<td>380</td>
<td>440</td>
</tr>
<tr>
<td>ACFP</td>
<td>439</td>
<td>476</td>
</tr>
<tr>
<td>AmCyan</td>
<td>458</td>
<td>489</td>
</tr>
<tr>
<td>AcGFP</td>
<td>475</td>
<td>505</td>
</tr>
<tr>
<td>ZsGreen</td>
<td>493</td>
<td>505</td>
</tr>
<tr>
<td>Vitality .RTM. hrGFP</td>
<td>500</td>
<td>506</td>
</tr>
<tr>
<td>Monster Green</td>
<td>505</td>
<td>515</td>
</tr>
<tr>
<td>EYFP</td>
<td>512</td>
<td>529</td>
</tr>
<tr>
<td>ZsYellow</td>
<td>529</td>
<td>539</td>
</tr>
<tr>
<td>DsRed-Express</td>
<td>557</td>
<td>579</td>
</tr>
<tr>
<td>DsRed2</td>
<td>563</td>
<td>582</td>
</tr>
<tr>
<td>DsRed</td>
<td>558</td>
<td>583</td>
</tr>
<tr>
<td>AsRed2</td>
<td>576</td>
<td>592</td>
</tr>
<tr>
<td>HcRed1</td>
<td>588</td>
<td>618</td>
</tr>
</tbody>
</table>

[00248] Non-limiting examples of fluorescent proteins that can be operably-linked to an ER-MAM-associated protein include, e.g., photoproteins, such as, e.g., aequorin; obelin; Aequorea fluorescent proteins, such, e.g., green fluorescent proteins (GFP, EGFP, AcGFP.sub.1), cyan fluorescent proteins (CFP, ECFP), blue fluorescent proteins (BFP, EBFP), red fluorescent proteins (RFP), yellow fluorescent proteins (YFP, EYFP), ultraviolet fluorescent protein (GFPuv), their fluorescence-enhancement variants, their peptide destabilization variants, and the like; coral reef fluorescent proteins, such, e.g., Discosoma red fluorescent proteins (DsRed, DsRedl, DsRed2, and DsRed-Express), Anemonea red fluorescent proteins (AsRed and AsRed2), Heteractis far-red fluorescent proteins (HcRed, HcRedl), Anemonea cyan fluorescent proteins (AmCyan, AmCyanl), Zoanthus green fluorescent proteins (ZsGreen, ZsGreenl), Zoanthus yellow fluorescent proteins (ZsYellow, ZsYellowl), their fluorescence-enhancement variants, their peptide destabilization variants, and the like; Renilla reniformis green fluorescent protein (Vitality hrGFP), its fluorescence-enhancement variants, its peptide destabilization variants, and the like; and Great Star Coral
fluorescent proteins, such, e.g., Montastrea cavernosa fluorescent protein (Monster Green.RTM. Fluorescent Protein), its fluorescence-enhancement variants, its peptide destabilization variants, and the like. It is apparent to one skilled in the art that these and a variety of other fluorescent proteins can be useful as a fluorescent protein in aspects of the invention, see, e.g., Jennifer Lippincott-Schwartz & George H. Patterson, Development and Use of Fluorescent Protein Markers in Living Cells, 300(5616) Science 87-91 (2003); and Jin Zhang et al, 3(12) Nat. Rev. Mol. Cell. Biol. 906-918 (2002).

[00249] It is apparent to one skilled in the art that these and many other fluorescent proteins, including species orthologs and paralogs of the herein described naturally occurring fluorescent proteins as well as engineered fluorescent proteins can be useful as a fluorescent protein disclosed in aspects of the present specification. ER-MAM-associated proteins disclosed in the present specification containing, in part, such fluorescent proteins can be prepared and expressed using standard methods see, e.g., Living Colors.RTM. User Manual PT2040-1 (PRI1Y691), BD Biosciences-Clontech, (Nov. 26 2001); BD Living Colors.TM. User Manual Volume II: Reef Coral Fluorescent Proteins, PT3404-1 (PR37085), BD Biosciences-Clontech, (Jul. 17, 2003); Monster Green Florescent Protein pHMCFP Vector, TB320, Promega Corp., (May, 2004); and Vitality hrGFP Mammalian Expression Vectors, Instruction Manual (rev. 064007g), Stratagene, Inc. Expression vectors suitable for bacterial, mammalian and other expression of fluorescent proteins are available from a variety of commercial sources including BD Biosciences Clontech (Palo Alto, Calif); Promega Corp. (Madison, Wis.) and Stratagene, Inc. (La Jolla, Calif).

Indicators of ER-MAM Integrity as Measured by Lanthanide Donor Complex Luminescence

[00250] A luminescence resonance energy transfer (LRET) pair comprises a lanthanide donor complex and an acceptor where the overlap between the emissions spectrum of the lanthanide donor complex and the absorbance spectrum of the acceptor is sufficient to enable LRET.

[00251] Aspects of the present invention can rely on a recombinant ER-MAM-associated protein which contains a donor fluorophore comprising a lanthanide donor complex. In other aspects, a donor fluorophore is a lanthanide donor complex. An ER-MAM-associated protein comprising a lanthanide donor complex exploits the luminescent properties of lanthanides, which are their long, millisecond to submillisecond lifetimes, narrow and multiple emission bands in the visible spectrum, and unpolarized emission.
[00252] A lanthanide donor complex includes a lanthanide ion such as, without limitation, a terbium ion, europium ion, samarium ion or dysprosium ion. Lanthanide ions, or "rare earth" elements, are a group of elements whose trivalent cations emit light at well-defined wavelengths and with long decay times. Lanthanides include, without limitation, elements with atomic numbers 57 through 71: lanthanide (La); cerium (Ce); praseodymium (Pr); neodymium (Nd); promethium (Pm); samarium (Sm); europium (Eu); gadolinium (Gd); terbium (Tb); dysprosium (Dy); holmium (Ho); erbium (Er); thulium (Tm); ytterbium (Yb); and lutetium (Lu). Lanthanides can further include, without limitation, yttrium (Y; atomic number 39) and scandium (Sc; atomic number 21).

[00253] A lanthanide-binding site useful in a lanthanide donor complex can be a peptide or peptidomimetic, such as, e.g., an EF-hand motif. As used herein, the term "EF-hand motif" means two a-helices flanking the coordination site of an EF-hand motif. A variety of naturally occurring EF-hands are known in the art, as described, e.g., Hiroshi Kawasaki and Robert H. Kretsinger, Calcium-Binding Proteins 1: EF-Hands, 1(4) Protein Profile 343-517 (1994); and Susumu Nakayama and Robert H. Kretsinger, Evolution of the EF-Hand Family of Proteins, Annu. Rev. Biophys. Biomol. Struct. 473-507 473-507 (1994); Hiroshi Kawasaki et al., Classification and Evolution of EF-Hand Proteins, 11(4) Biometals 277-295 (1998); and Yubin Zhou et al., Prediction of EF-Hand Calcium-Binding Proteins and Analysis of Bacterial Proteins 65(3) Proteins 643-655 (2006).

Indicators of Altered ER-MAM Integrity: Cell death

[00254] In another aspect, the invention relates to the correlation of Alzheimer's disease with an indicator of altered ER-MAM integrity involving cell death. In one aspect, the invention provides a method for determining whether a test compound is capable of treating Alzheimer's disease by comparing a cellular response to an apoptogenic stimulus, where such response is an indicator of altered ER-MAM integrity as provided herein. Altered mitochondrial physiology can be involved in programmed cell death (Zamzami et al., Exp. Med. 182:367-77, 1995; Zamzami et al, Exp. Med. 181:1661-72, 1995; Marchetti et al, Cancer Res. 56:2033-38,1996; Monaghan et al, J. Histochem. Cytochem. 40:1819-25, 1992; Korsmeyer et al, Biochim. Biophys. Act. 1271:63, 1995; Nguyen et al, Biol. Chem. 269:16521-24, 1994). Thus, changes in mitochondrial physiology can be important mediators of cell death. Altered mitochondrial function, as can be used for determining whether a test compound is capable of treating Alzheimer's disease according to the present disclosure, can
therefore increase the threshold for induction of cell death by an apoptogen. A variety of apoptogens are known to those familiar with the art (see, e.g., Green et al, 1998 Science 281:1309 and references cited therein).

[00255] In one embodiment of the subject invention method wherein the indicator of altered ER-MAM integrity is a cellular response to an apoptogen, cells in a biological sample that are suspected of undergoing apoptosis can be examined for morphological, permeability or other changes that are indicative of an apoptotic state. For example by way of illustration and not limitation, apoptosis in many cell types can cause altered morphological appearance such as plasma membrane blebbing, cell shape change, caspase activation, translocation of cell membrane phosphatidylserine from the inner to the outer leaflet of the plasma membrane, loss of substrate adhesion properties or other morphological changes that can be readily detected by a person having ordinary skill in the art, for example by using light microscopy. In one embodiment, cell death can be measured with a "live-dead" assay (e.g. living cells are green whereas dead cells are red).

[00256] A person having ordinary skill in the art will readily appreciate that there can be other suitable techniques for quantifying apoptosis, and such techniques for purposes of determining an indicator of ER-MAM integrity that is a cellular response to an apoptogenic stimulus are within the scope of the methods provided by the present invention.

Indicators of Altered ER-MAM Integrity: APP Cleavage or β-Secretase Activity

[00257] Any known marker or correlate to AD can be used as a marker of altered ER-MAM integrity. While not wishing to be bound to theory, inhibition of β-secretase activity is thought to inhibit production of β amyloid β peptide (Aβ). Reduction of APP cleavage at the β-secretase cleavage site compared with an untreated or inactive control can be used to determine inhibitory activity. Methods for determining β-secretase activity are known in the art. Exemplary systems include, but are not limited to assay systems are described in U.S. Pat. No. 5,942,400. Thus, in one embodiment, the extent rate or amount cleavage of APP at the β-secretase cleavage site can be used as a marker of ER-MAM integrity. Assays that demonstrate inhibition of β-secretase-mediated cleavage of APP can utilize any of the known forms of APP (see, for example, U.S. Pat. No. 5,766,846 and also Hardy, 1992, Nature Genet. 1:233-234).

Indicators of Altered ER-MAM Integrity: Reduced Glucose Metabolism

- 68 -
Reduced glucose utilization and deficient energy metabolism occur in the pathogenesis of AD (Castellani, R. et al. Role of mitochondrial dysfunction in Alzheimer’s disease. J. Neurosci. Res. 70, 357-360 (2002)). Methods for measuring glucose metabolism in a biological sample are well known in the art (e.g. glucose-6-phosphate phosphatase can be assayed by established procedures (Vance and Vance, 1988).

**Indicators of Altered ER-MAM Integrity: Cholesterol Content**

Cholesterol is selectively reduced an AD "double-transgenic" (i.e. mutations in both APP and PS1) mouse model (Yao et al. (2008) Neurochem. Res. in press).

Methods for measuring cholesterol content of a biological sample are well known in the art (e.g. fillipin staining).

**Correlation of Apolipoprotein Genotype**

In humans, there are three alleles of apolipoprotein E: ApoE2, ApoE3, and ApoE4. Individuals harboring at least one ApoE4 allele are at risk for developing sporadic AD (SAD). Like PS1 and PS2, ApoE4 is a ER-MAM-localized protein. The results described herein show that the mitochondrial maldistribution phenotype, as well as the increase in communication between the ER and mitochondria (both indicators of altered ER-MAM integrity) are correlated to the ApoE4 genotype. Specifically, cells with E3/E3 have normal ER-MAM content, whereas those with E3/E4 have increased communication between the ER and mitochondria, irrespective of whether or not the cells harbor a presenilin mutation (e.g. cells with a PS1 mutation and an E3/E3 genotype have normal communication between the ER and mitochondria and normal mitochondrial distribution, whereas PS1 cells with E3/E4 have increased communication between the ER and mitochondria and altered mitochondria). Similarly, the amount of communication between the ER and mitochondria in E3/E4 brain tissue from FAD<sup>PS1</sup> or FAD<sup>PS2</sup> patients is increased compared to that in E3/E3 brain tissue from FAD<sup>PS1</sup> or FAD<sup>PS2</sup> patients. This result explains the role of ApoE in the pathogenesis of AD, and connects the familial and sporadic forms of the disease into one conceptual framework.

The finding that presenilin is a ER-MAM-associated protein, that the amount of communication between the ER and mitochondria is increased in FAD<sup>PS1</sup> or FAD<sup>PS2</sup> cells, and that ApoE4 allele status can affect ER-MAM integrity show that the fundamental problem in both FAD and SAD is altered ER-MAM integrity. Thus in one aspect, the invention described herein provides a method for determining whether a subject
has an ApoE3/E4 genotype, the method comprising, obtaining a biological sample from an individual suspected of having Alzheimer's disease, measuring an indicator of ER-MAM integrity in the biological sample and comparing the indicator of ER-MAM integrity in the biological sample of step to the indicator of ER-MAM integrity in a control sample wherein, a change in the indicator of ER-MAM integrity measured in the biological sample compared to the control sample indicates that the subject has an ApoE3/E4 genotype. In another aspect, the invention described herein provides a method for determining whether a subject has an ApoE4/E4 genotype, the method comprising, obtaining a biological sample from an individual suspected of having Alzheimer's disease, measuring an indicator of ER-MAM integrity in the biological sample and comparing the indicator of ER-MAM integrity in the biological sample of step to the indicator of ER-MAM integrity in a control sample wherein, a change in the indicator of ER-MAM integrity measured in the biological sample compared to the control sample indicates that the subject has an ApoE4/E4 genotype.

**Biological Samples**

[00263] Biological samples can comprise any tissue or cell preparation in which at least one candidate indicator of altered ER-MAM integrity can be detected, and can vary in nature accordingly, depending on the indicator(s) of ER-MAM integrity to be compared. Biological samples can be provided by obtaining a blood sample, biopsy specimen, tissue explant, organ culture or any other tissue or cell preparation from a subject or a biological source. The subject or biological source can be a human or non-human animal, a primary cell culture or culture adapted cell line including but not limited to genetically engineered cell lines. For example, suitable biological samples for diagnosing Alzheimer's disease include cells obtained in a non-invasive manner. Examples include, but are not limited to an AD model cell, a neuron, a fibroblast, a skin biopsy, a blood cell (e.g. a lymphocyte), an epithelial cell and biological materials found in urine sediment. In some embodiments, for example embodiments of methods for screening for screening of compounds, yeast cells, fungi and other eukaryotic cells (e.g. plant cells) can also be used.

[00264] AD model disease cells suitable for use with the methods described herein include, but are not limited to, human skin fibroblasts derived from patients carrying FAD-causing presenilin mutations, mouse skin fibroblasts, cultured embryonic primary neurons, and any other cells derived from PSI-knock out transgenic mice (containing null mutation in the PSI gene), cells having AD-linked familial mutations, cells having genetically associated AD allelic variants, cells having sporadic AD, or cells having mutations associated with
sporadic AD. In some embodiments, for example embodiments of methods for screening for screening of compounds, yeast cells, fungi and other eukaryotic cells (e.g. plant cells) can also be used.


[00268] A tissue can be treated to release one or more individual component cell or cells and the cells can then be treated to release the individual component organelles and so on. Partitioned samples (such as in cells, organelles, cellular fractions) can serve as a protein source for discrimination in 2-D gels and any further methodologies described herein as well as any methodologies known to one skilled in the art.

[00269] In the case of a tissue, a tissue sample can be obtained and prepared for separation of the proteins therein using a method that provides suitable levels of discrimination of the proteins of the cell. The proteins can be obtained by any of a variety known means, such as enzymatic and other chemical treatment, freeze drying the tissues, with or without a solubilizing solution, repeated freeze/thaw treatments, mechanical treatments, combining a mechanical and chemical treatment and using frozen tissue samples and so on.

[00270] To provide a more specific origin of protein, specific kinds of cells can be purified from a tissue using known materials and methods. To provide proteins specific for an organelle, the organelles can be partitioned, for example, by selective digestion of unwanted organelles, density gradient centrifugation or other forms of separation, and then the organelles can be treated to release the proteins therein and thereof.

[00271] Lipid rafts are lipid subdomains that are enriched in cholesterol, and are thicker than surrounding membrane lipids. Moreover, they are detergent insoluble and are resistant to the detergent Triton X-100 (TX-100). The results described herein show that ER-MAM is lipid TX-100-resistant, and is cholesterol-rich. Without being bound by theory, ER-MAM in subjects having, or at risk of having AD can have altered structure as compared to normal ER-MAM (hence the increase in ER-MAM content in or FAD^{PS1} and or FAD^{PS2} patients). This difference can be exploited both in diagnosis and treatment by using indicators of ER-MAM integrity to determine ER-MAM structure. Thus, in one aspect, the invention provides methods for diagnosing AD in a subject or methods for determining whether a test compound is capable of treating Alzheimer's disease wherein the methods comprise characterization of subcellular membranes or subcellular fractionation.

[00272] A variety of methods have been developed aimed at the isolation of one or more subcellular fractions. For example, subcellular fractionation using density gradients and zonal centrifuges is known to one skilled in the art (Anderson, "The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis" National Cancer
Institute Monograph 21, 1966). Methods for isolating ER-MAM are also known to those skilled in the art (Vance, 1990; and see Example 3)

A crude protein preparation also can be exposed to a treatment that partitions the proteins based on a common property, such as size, subcellular location and so on. For example, the crude lysate can be partitioned prior to high-resolution separation of the proteins to reduce the number of proteins for ultimate separation and to enhance discrimination. Thus, the crude lysate can be fractionated by chromatography. Such a preliminary treatment can be useful when a sample is known to contain one or more abundant proteins. Removing abundant proteins can enhance the relative abundance of minor species of proteins that can be analyzed.

Multiple preliminary fractionation steps can be practiced, such as, using multiple chromatography steps, with the chromatography steps being the same or different, or multiple extraction or other partitioning steps. Suitable chromatography methods include those known in the art, such as immunoaffinity, size exclusion, lectin affinity and so on.

Screening Methods and Compound Libraries

The invention also provides methods useful for identifying compounds or agents which are capable of treating Alzheimer's disease, an in certain embodiments, more generally a neurodegenerative diseases that have altered ER-MAM, in a subject. Generally, test compounds are selected if they can reverse an indicator of ER-MAM in a biological sample, model AD cell or animal-model to a state or condition or level comparable to a wild-type or normal cell or animal. In one embodiment, a test compound can be examined for an ability to increase or a decrease an indicator of ER-MAM integrity in a cell.

Suitable biological samples for identifying compounds or agents which are capable of treating Alzheimer's disease can comprise any tissue or cell preparation in which at least one candidate indicator of altered ER-MAM integrity can be detected, and can vary in nature accordingly, depending on the indicator(s) of ER-MAM integrity to be compared. Biological samples can be provided by obtaining a blood sample, biopsy specimen, tissue explant, organ culture or any other tissue or cell preparation from a subject or a biological source. The subject or biological source can be a human or non-human animal, a primary cell culture or culture adapted cell line including but not limited to genetically engineered cell lines. For example, suitable biological samples for diagnosing Alzheimer's disease include cells obtained in a non-invasive manner. Examples include, but are not limited to a neuron, a
fibroblast, a skin biopsy, a blood cell (e.g. a lymphocyte), an epithelial cell and biological materials found in urine sediment.

[00277] AD model disease cells suitable for use with the methods described herein include, but are not limited to, human skin fibroblasts derived from patients carrying FAD-causing presenilin mutations, mouse skin fibroblasts, cultured embryonic primary neurons, and any other cells derived from PS1-knock out transgenic mice (containing null mutation in the PS1 gene), cells having AD-linked familial mutations, cells having genetically associated AD allelic variants, cells having sporadic AD, or cells having mutations associated with sporadic AD.


[00279] Genetically associated AD allelic variants include, but are not limited to, allelic variants of apolipoprotein E (e.g., APOE4) (Strittmatter, W. J. et al., *Proc. Natl. Acad. Sci. USA* 90:1977-1981 (1993)).


[00282] Examples of such compounds include, but are not limited to, small organic molecules including pharmaceutically acceptable molecules. Examples of small molecules include, but are not limited to, polypeptides, peptidomimetics, amino acids, amino acid analogs, nucleic acids, nucleic acid analogs, nucleotides, nucleotide analogs, organic or inorganic compounds (i.e., including heteroorganic and organometallic compounds) having a molecular weight of less than about 10,000 grams per mole, salts, esters, and other pharmaceutically acceptable forms of such compounds. Examples of other compounds that can be tested in the methods of this invention include polypeptides, antibodies, nucleic acids, and nucleic acid analogs, natural products and carbohydrates.

[00283] A compound can have a known chemical structure but not necessarily have a known function or biological activity. Compounds can also have unidentified structures or be mixtures of unknown compounds, for example from crude biological samples such as plant extracts. Large numbers of compounds can be randomly screened from
chemical libraries, or collections of purified chemical compounds, or collections of crude extracts from various sources. The chemical libraries can contain compounds that were chemically synthesized or purified from natural products. Methods of introducing test compounds to cells are well known in the art.

[00284] Those having ordinary skill in the art will appreciate that a diverse assortment of compound libraries can be prepared according to established procedures, and tested for their influence on an indicator of altered ER-MAM integrity. The test compounds can be obtained using any of the numerous approaches in combinatorial library methods known in the art (see Lam K S. Anticancer Drug Des. 12:145-67 (1997)). Such compound libraries are also available from commercial sources such as ComGenex (U.S. Headquarters, South San Francisco, Calif), Maybridge (Cornwall, UK), and SPECS (Rijswijk, Netherlands), ArQule, Tripos/PanLabs, ChemDesign and Pharmacopoeia.

[00285] Therapeutic agents or combinations of agents suitable for the treatment or prevention of AD can be identified by screening of candidate agents on normal, AD or hybrid cells constructed with patient mitochondria. The invention also provides methods of identifying an agent suitable for treating a subject suspected of being at risk for having AD by comparing the level of at least one indicator of altered ER-MAM integrity, in the presence and absence of a candidate compound, to determine the suitability of the agent for treating AD. The compounds identified in the screening methods of this invention can be novel or can be novel analogs or derivatives of known therapeutic agents.

Expression of Presenilin

[00286] In one aspect, the invention described herein provides methods for determining whether a test compound is capable of treating Alzheimer's disease. In one embodiment, the method comprises overexpressing presenilin or reducing presenilin expression with shRNA technology, contacting a cell or biological sample with a test compound, measuring an indicator of ER-MAM integrity in the cell, and comparing the indicator of mitochondria-associated membrane integrity measured in the cell or biological sample with an indicator of ER-MAM integrity measured in a control cell or biological sample that has not been contacted with a test compound, wherein an increase or decrease in the indicator of mitochondria-associated membrane integrity measured in the cell or biological sample relative to the indicator of mitochondria-associated membrane integrity
measured in the control cell or biological sample indicates that the test compound is capable of treating Alzheimer's disease.

Classification of AD

[00287] The present invention provides compositions and methods that are useful in pharmacogenomics, for the classification of a subject or patient population without the use of a genetic test. In one embodiment, for example, such classification can be achieved by identification in a subject or patient population of one or more distinct profiles of at least one indicator ER-MAM integrity that correlate with AD. Such profiles can define parameters indicative of a subject's predisposition to develop AD, and can further be useful in the identification of new subtypes of AD. In another embodiment, correlation of one or more traits in a subject with at least one indicator of altered ER-MAM integrity can be used to gauge the subject's responsiveness to, or the efficacy of, a therapeutic treatment.

[00288] As described herein, determination of levels of at least one indicator of altered ER-MAM integrity can also be used to classify a AD patient population (i.e., a population classified as having AD by independent criteria). In another embodiment of the invention, determination of levels of at least one indicator of altered ER-MAM integrity in a biological sample from a AD subject can provide a useful correlative indicator for that subject. An AD subject so classified on the basis of levels of at least one indicator of altered ER-MAM integrity can be monitored using AD clinical parameters, such that correlation between levels of at least one indicator of altered ER-MAM integrity and any clinical score used to evaluate AD can be monitored as a useful marker with which to correlate the efficacy of any candidate therapeutic agent being used in AD subjects.

Recombinant Expression Vectors and Host Cells

[00289] The recombinant expression vectors for expression of polypeptides of this invention in prokaryotic or eukaryotic cells can be designed. For example, polypeptide of this invention can be expressed in bacterial cells such as insect cells (e.g., using baculovirus expression vectors), yeast cells, amphibian cells, or mammalian cells. Suitable host cells are well known to one skilled in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, see chapters 16 and 17 of Sambrook, J., Fritsh, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.
A number of these methodologies can also be applied in vivo, systemically or locally, in a complex biological system such as a human. For example, increased copy number of nucleic acids encoding ER-MAM-associated proteins in expressible from (by DNA transfection), can be employed.

Animal Models

Once an test compound is identified to be able, modulate an indicator of ER-MAM integrity, modulate the ratio of perinuclear mitochondria to non-perinuclear mitochondria is a cell, modulate the amount of communication between the ER and mitochondria in a biological sample, modulate the ratio punctate to non-punctate mitochondria in a cell, modulate the conversion of phosphatidylycerine to phosphatidylethanolamine in a biological sample, modulate the amount of cell survival in a cell contacted with cinnamycin, modulate the association of ER-MAM-associated proteins (e.g. Diacylglycerol-O-acyltransfease 2 (DGAT2) and stearoyl-CoA desaturase 1 (SCD1)), modulate the amount of one or more reactive oxygen species in a cell, or modulate an indicator of mitochondria-associated integrity in a cell, the agent can be tested for its ability to treat Alzheimer's disease in animal models. Animal models useful in testing the such compounds include those expressing elevated levels of Aβ, demonstrating an enhanced amount of Aβ deposits, and/or increased number or size of β amyloid plaques as compared with control animals. Suitable animal models include, but are not limited to transgenic mammals, including but not limited to ApoE4 mice (e.g. mice having human a ApoE4 gene or a knock-in to "humanize" the mouse ApoE gene).

Compromised ER-MAM Integrity in Neurodegenerative Diseases and Disorders

[00293] In a further aspect, the diagnostic methods disclosed herein can be used for determining whether a subject has, or is at risk of having a neurodegenerative disease or disorder. In one embodiment, the neurodegenerative disease or disorder is Alzheimer’s disease. In another aspect, the screening methods disclosed herein can be used to identify a compound useful in the treatment, prevention or reduction of a neurodegenerative disease or disorder. In one embodiment, the neurodegenerative disease or disorder is Alzheimer’s disease.

[00294] In one embodiment, an indicator of altered ER-MAM integrity in neurodegenerative disease or disorder, or a cell from a subject suspected for having neurodegenerative disease or disorder is any of: (a) an altered ratio of perinuclear to non-perinuclear mitochondria as compared to a normal control cell; (b) an altered ratio of punctate mitochondria to non-punctate mitochondria as compared to a normal control cell; (c) an altered amount of mitochondria at extremities of cells as compared to a normal control cell; (d) altered mitochondrial movement as compared to a normal control cell; (e) an altered amount of free radial production as compared to a normal control cell; (f) altered phosphatidylserine conversion to phosphatidylethanolamine as compared to a normal control cell; (g) altered mitochondrial calcium levels as compared to a normal control cell; (h) altered cytosolic calcium levels as compared to a normal control cell; (i) an altered localization of ER-MAM associated proteins to ER-MAM as compared to a normal control cell; (j) altered total ER-MAM as compared to a normal control cell; (k) an altered activity of one or more non-presenilin ER-MAM-association proteins as compared to a normal control cell; (l) an altered amount of functional presenilin in ER-MAM as compared to a normal control cell; (m) an altered amount of association between ER-MAM associated proteins as compared to a normal control cell; (n) an altered sensitivity to cinnamycin induced cell death as compared to a normal control cell; (o) an altered cholesterol content in ER-MAM as compared to a normal control cell; (p) altered ATP biosynthesis in mitochondria as compared to a normal control cell; (q) an altered communication between ER and mitochondria as compared to a normal control cell; (r) an altered amount cell death in response to an apoptogen as compared to a normal control cell; (s) altered ACATI activity as compared to a normal control cell; (t) altered cholesterol ester synthesis as compared to a normal control cell; (u) altered lipid droplet formation as
compared to a normal control cell; or (v) an altered ratio of Abeta42 to Abeta40 in isolated ER-MAM as compared to a normal control cell.

[00295] Exemplary neurodegenerative diseases or disorders include, but are not limited to, Alexander disease, Alper's disease, Alzheimer's disease (Sporadic and Familial), Amyotrophic lateral sclerosis, Ataxia telangiectasia, Batten disease (also known as Spielmeyer-Vogt-Sjogren-Batten disease), Bovine spongiform encephalopathy (BSE), Canavan disease, Cockayne syndrome, Corticobasal degeneration, Creutzfeldt-Jakob disease, Huntington disease, HIV-associated dementia, Kennedy's disease, Krabbe disease, Lewy body dementia, Machado-Joseph disease (Spinocerebellar ataxia type 3), Multiple sclerosis, Multiple System Atrophy, Parkinson disease, Pelizaeus-Merzbacher Disease, Pick's disease, Primary lateral sclerosis, Refsum's disease, Sandhoff disease, Schilder's disease, Schizophrenia, Spielmeyer-Vogt-Sjogren-Batten disease (also known as Batten disease), Spinocerebellar ataxia (multiple types with varying characteristics), Spinal muscular atrophy, Steele-Richardson-Olszewski disease, Tabes dorsalis, Angelman syndrome, Autism, Fetal Alcohol syndrome, Fragile X syndrome, Tourette's syndrome, Prader-Willi syndrome, Sex Chromosome Aneuploidy in Males and in Females, William's syndrome, Smith-Magenis syndrome, 22q Deletion, or any combination thereof.

[00296] The following examples illustrate the present invention, and are set forth to aid in the understanding of the invention, and should not be construed to limit in any way the scope of the invention as defined in the claims which follow thereafter.

EXAMPLES

[00297] Example 1: Presenilins are negative regulators of ER-mitochondrial communication

[00298] Presenilin-1 (PS1) and -2 (PS2), and γ-secretase activity, are enriched in a subcompartment of the endoplasmic reticulum (ER) which physically and functionally interacts with mitochondria, called ER membranes associated with mitochondria (MAM). As described herein, MAM displays the features of an intracellular lipid raft, and that the absence of presenilins upregulates the communication between ER and mitochondria, as measured by two key biochemical assays of MAM behavior, phospholipid transport and cholesteryl ester synthesis. Cells lacking presenilins also displayed a significant increase in the physical association of these two compartments. The results described herein demonstrate
that presenilins are negative regulators of ER-mitochondrial communication, and that this upregulation plays a key role in the pathogenesis of Alzheimer disease.

[00299] Alzheimer disease (AD) is a late onset neurodegenerative disorder characterized by progressive neuronal loss, especially in the cortex and the hippocampus (Goedert and Spillantini (2006) Science 314, 777-781). The two main histopathological hallmarks of AD are the accumulation of neurofibrillary tangles, consisting mainly of hyperphosphorylated forms of the microtubule-associated protein tau, and of extracellular neuritic plaques, consisting mainly of β-amyloid (Aβ) species (predominantly Aβ40 and Aβ42), a 4-kDa peptide derived from the cleavage of the amyloid precursor protein (APP) by β- and γ-secretases (Goedert and Spillantini (2006) Science 314, 777-781). The vast majority of AD is sporadic, but mutations in PS1, and PS2, as well as in APP, have been identified in the familial form. PS1 and PS2 are aspartyl proteases that are components of the γ-secretase complex that processes a number of membrane-bound proteins, including APP.


[00301] As described herein, both PS1 and PS2, and γ-secretase activity itself, are enriched in the MAM. We now show that MAM is a DRM displaying the characteristics of an intracellular lipid raft. Moreover, the results described herein show that the loss of presenilins affects MAM structure profoundly and increases functions associated with MAM, suggesting that presenilins act as negative regulators of ER-mitochondrial communication.

[00302] The differentially lower density of MAM in a gradient as compared to that of bulk ER or mitochondria, as described herein, led us to speculate that MAM has a composition similar to that of lipid rafts (Simons and Vaz (2004) Annu. Rev. Biophys. Biomol. Struct. 33, 269-295).

[00303] Purified MAM from mouse tissues was therefore incubated with and without Triton-X 100 (TX100), and loaded both samples onto a Percoll gradient under the same conditions used for its initial isolation. Fig. 37 shows the fractionation of mouse tissues to isolate MAM. The TX100-treated MAM sample was fundamentally intact and migrated to the identical position in the gradient as did the untreated sample, consistent with the behavior of a DRM (Fig. 38A).

[00304] To separate LR from other cell contents, TX100-treated and control MAM fractions were loaded onto a sucrose gradient (Ostrom and Liu (2007) Meth. Mol. Biol. 400, 459-468), and analyzed fractions by Western blotting to detect known MAM markers: Pemt (phosphatidylethanolamine N-methyltransferase) (Vance (1990) J. Biol. Chem. 265, 7248-7256), Vdac1 (voltage-dependent anion channel 1) (Hayashi et al., (2009) Trends Cell Biol. 19, 81-88), and PS1 (Fig. 38B). The proteins migrated at similar positions in the lower density fractions, and the migration pattern was unaffected by detergent treatment (Fig. 38B). Importantly, MAM was not contaminated with LR/DRMs from plasma membrane (PM), as Src, a marker for PM LR/DRMs (Morrow and Parton (2005) Traffic 6, 725-740), was observable in sucrose gradient fractions from purified PM, but not from the crude mitochondrial fraction (CM) from which the MAM fraction was derived (Fig. 38C).

Moreover, the cholesterol content of mouse brain MAM was higher than that found in the cytoplasm, mitochondria, bulk ER, and total PM, and was comparable to that of LR from PM (Simons and Vaz (2004) Annu. Rev. Biophys. Biomol. Struct. 33, 269-295) (Fig. 39A). By
contrast, purified mitochondria and bulk ER from the bottom of the gradient behaved like detergent-soluble fractions (Fig. 40), indicating the absence of DRMs, as expected (Zheng et al., (2009) J. Lipid Res. 50, 988-998). Fig. 40 shows that neither bulk ER nor mitochondria are detergent-resistant membranes. These results show that presenilins and γ-secretase activity resides in a lipid rafts and indicate that MAM is an intracellular lipid raft-like domain that may recruit and orient various signaling proteins needed to regulate cross-talk between ER and mitochondria.

[00305] MAM/mitochondria markers, such as VDAC (Fig. 38B) or calnexin (Hayashi et al., (2009) Trends Cell Biol. 19, 81-88; Foster and Chan (2007) Subcell. Biochem. 43, 35-47) have been found in PM because, apart from the lack of appropriate markers to detect MAM, most LR isolation methods do not separate PM from intracellular membranes (Macdonald and Pike (2005) J. Lipid Res. 46, 1061-1067); such PM raft preparations will therefore be cross-contaminated with MAM. Moreover, several authors have described or suggested the existence of intracellular rafts in the ER or mitochondria (Browman et al., (2006) J. CellSci. 119, 3149-3160; Mellgren (2008) J. Biochem. Biophys. Methods 70, 1029-1036; Martinez-Abundis et al., (2009) FEBS J. 276, 5579-5588). These intracellular LR/DRMs are in fact MAM (Hayashi and Fujimoto (2010) Mol. Pharmacol. 11, 517-528). Conversely, crude mitochondrial preparations contain MAM, and can be misinterpreted to suggest that mitochondria contain LR (Martinez-Abundis et al., (2009) FEBS J. 276, 5579-5588), when in fact they do not (Zheng et al., (2009) J. Lipid Res. 50, 988-998) (Fig. 40).


[00307] As MAM is a LR/DRM, the regulation of cholesterol metabolism may be an important determinant of its structure and function. Acyl-CoA:cholesterol acyltransferase (ACAT), which catalyzes the conversion of free cholesterol to cholesteryl esters, is enriched in MAM (Rusinol et al., (1994) J. Biol. Chem. 269, 27494-27502). ACAT controls the equilibrium between membrane-bound free cholesterol and cholesteryl esters stored in cytoplasmic lipid droplets (23). ACAT1, the predominant ACAT isoform in brain, was
confirmed to not only be more abundant in MAM compared to bulk ER and mitochondria (Fig. 39B, inset), but also had an approximately 3-fold higher enzymatic activity (Fig. 39B).

[00308] In order to determine if presenilins play a role in regulating MAM function, mouse embryonic fibroblasts (MEFs) lacking PSI (PS1-KO), PS2 (PS2-KO), or both proteins (DKO) were analyzed (Herreman et al., (2000) Nat. Cell Biol. 2, 461-462), focusing first on cholesterol synthesis and ACAT activity. Compared to WT MEFs, the mutant lines showed increased steady state levels of total and free cholesterol (Fig. 39C) (Grimm et al, (2005) Nat. Cell Biol. 7, 1118-1123). Further analysis showed higher free cholesterol contents in mutant vs. WT MEFs, but more importantly, the relative differences in the content of cholesteryl esters (CE) were even greater, with up to ~5-fold more CE in DKO than in WT MEFs (Fig. 39C). Notably, significantly higher ACAT activity in DKO MEFs was observed, measured both in cultured cells in vivo (Fig. 39E) and in isolated MAM in vitro (Fig. 39F). Numerous CE-containing lipid droplets in the DKO cells were detected that were absent in control MEFs (Fig. 39D).

[00309] Upon labeling the cells with 3H-cholesterol to monitor its conversion by ACAT into 3H-cholesteryl esters, we found significantly higher ACAT activity in the PsI-mutant MEFs (up to ~2.5-fold over controls) (Figure 391). Both presenilin isoforms contributed to CE synthesis, as the average increase in CE content was far more pronounced in the PsI+Ps2 DKO MEFs (145% greater than control) than in either individual knockout alone (96% and 63% greater than control in PsI- and Ps2-KO, respectively) (Figure 391). Because CE synthesis in the PsI-KO cells was higher than in the Ps2-KOs, and because PSI plays a more significant role than PS2 in FAD (Jayadev et al., 2010), CE synthesis was also examined in immortalized mouse MEFs in which PsI expression had been knocked down (PsI-KD) (Figure 17). There was a ~3-fold increase in the kinetics of CE formation in PsI-KD cells vs control (Figure 39G, left panel). Notably, significant increases in CE synthesis were detected in PS-mutant FAD cells (~1.8-fold higher than controls), and equally strikingly, in SAD cells as well (~1.7-fold higher) (Figure 39H).

[00310] Consistent with the increase in CE, numerous structures that appeared to be lipid droplets were observed in electron microscopic images of DKO, but not control, MEFs (asterisks in Figure 44A). The presence of lipid droplets was examined after staining cells with HCS LipidTox GreenTM (Figure 44B) and Oil Red O (Figure 48). Whereas the LipidTox stain in WT MEFs was diffuse, the PS-mutant MEFs contained numerous discrete LipidTox-positive droplets (~5-fold greater signal) (Figure 44B; see also Figure 49). Similar
results were also obtained with the Psl-KD cells (Figure 44C); importantly, the increase in lipid droplets in Psl-KD cells (~5-fold over control) was rescued by overexpression of human WT PS1, but not of human PS1 harboring the A246E mutation found in many FAD patients (Figure 44C). Notably, significantly more lipid droplets were detected in FAD and SAD fibroblasts (20-30% of the cells were LipidTox-positive) vs controls (-3% positive) (Figure 44D and Figures 48 and 49). These observations may help explain the elevated lipids droplets found in fibroblasts (Pani et al., 2009) and neurons (Gomez-Ramos and Asuncion Moran, 2007) of SAD patients.

[00311] Taken together, the CE synthesis data support the view that presenilins play a key role in the regulation of this specific MAM function, an and involvement of presenilins in ER mitochondrial communication.


[00313] MAM is also required for the synthesis of most of the cell's phosphatidylethanolamine (PtdEtn) (Voelker (2000) Biochim. Biophys. Acta 1486, 97-107). Phosphatidylserine (PtdSer) is synthesized in the MAM via phosphatidylserine synthase 2 (Hayashi et al, (2009) Trends Cell Biol. 19, 81-88); PtdSer translocates to mitochondria, where it is converted to PtdEtn by phosphatidylserine decarboxylase; finally, PtdEtn translocates back to the MAM, where it is methylated by phosphatidylethanolamine methyltransferase (PEMT) (Vance (2008) J. Lipid Res. 49, 1377-1387) to generate
phosphatidylcholine. The trafficking of PtdSer from MAM to mitochondria is a recognized measure of MAM function (Voelker, 2005). Presenilin-mutant MEFs were incubated in medium containing 3H-serine and analyzed for the incorporation of the label into newly synthesized PtdSer and PtdEtn. The levels of both labeled species were highly elevated in the DKO MEFs compared to WT (Fig. 41A), suggesting upregulation of MAM-mitochondrial crosstalk.

**[00314]** Pulse-chase analysis was performed by incubating the MEFs with 3H-Ser for 1 hour, followed by a chase with cold serine (Fig. 41B). The incorporation of label into PtdSer during the pulse was higher in the mutant MEFs (time 0 in Fig. 41B). During the chase, the amount of 3H-PtdSer decreased and 3H-PtdEtn increased, consistent with the conversion of the former into the latter, with increased conversion rates in Psl-KO and DKO MEFs (up to 3-fold higher), again indicating increased MAM-mitochondrial communication.

**[00315]** While the increase in lipid synthesis in the pulse-chase was not observed by us in the Ps2-KO MEFs, Ps2 clearly contributes to phospholipid metabolism and MAM function, as lipid synthesis in the Psl+Ps2 double knockout was much more pronounced than in the Psl-knockout alone (Figure 41B). These results were confirmed in isolated MEF crude mitochondrial fractions (containing essentially only ER, MAM, and mitochondria (Area-Gomez et al, 2009)) (Figure 41C). The rate of phospholipid synthesis was also increased in Psl-KD cells (Figure 41E) and, importantly, in FAD and SAD fibroblasts (by -1.5- to 2-fold over controls) (Figure 41F, left panel).

**[00316]** As before, the synthesis of both phospholipid species was higher in the CM from mutant MEFs vs. control, confirming that the loss of presenilins resulted in upregulation of the interaction between ER and mitochondria. While the increase in lipid synthesis was least pronounced in the PS2-DKO MEFs, it is nevertheless clear that PS2, like PS1, contributes to ER-mitochondrial cross-talk, as the synthesis in the PS1+PS2 double knockout was much more pronounced than in the PS1 knockout alone.


**[00318]** Since some of the PtdEtn synthesized is exported to the inner leaflet of the plasma membrane (Vance, 2008), elevation of PtdEtn in the PM of mutant cells was examined. Accordingly, cells were treated with the antibiotic cinnamycin (Cin; also called
Ro09-0198) (Choung et al., 1988), a 19-aa cyclic peptide that forms a complex specifically with PtdEtn to induce pore formation in the PM, followed by rapid cell death, in a PtdEtn concentration dependent manner (Makino et al., 2003). PS-mutant MEFs were more Cin-sensitive than were controls (Fig. 41D), with the DKO MEFs up to 8-fold more sensitive (Figure 45A). Similarly, Psl-KD cells were 3-fold more Cin-sensitive than controls; as before, this sensitivity could be rescued by overexpression of human WT, but not A246E mutant, PS1 (Figure 45B, left panel). Notably, FAD and SAD cells were significantly (~3- to 5-fold) more Cin-sensitive than controls (Figure 45B, right panel). The presence of PtdEtn was visualized on the cell surface by staining cells with FL-SA-Ro (Figure 45C), a fluorescent-conjugated form of cinnamycin that binds to PtdEtn on the PM but does not initiate cell death (Emoto et al., 1996). In agreement with the Cin-sensitivity results, ~4 times as many FAD and SAD cells were stained with FL-SA-Ro as compared to controls (Figure 45C and Figure 50). Together with the CE data, these results show that MAM function is upregulated when PSs are mutated, via either knock-down or knock-out.

[00319] Electron microscopy of WT and DKO MEFs was performed to examine the association between ER and mitochondria to determine if ER mitochondrial contacts were physically altered (Fig. 42). Electron microscopy showed an increase in the length of mitochondrial-ER contacts (i.e. MAM) in PS-DKO as compared to WT MEFs (Figure 42). There were significantly more numerous "long" (50-200 nm; Figure 42C) and "very long" (>200 nm; Figure 42E) contacts in DKO MEFs than in WT MEFs (~5-fold and >10-fold, respectively; Figure 42F), whereas connections in WT MEFs were much shorter and more "punctate" (<50 nm; Figure 42A). Thus, the increased biochemical activity of MAM in PS-mutant cells correlated with an increased area of physical association between the two organelles.

[00320] The results described herein show that the increase in MAM-related functions in PS-mutant cells is due to upregulated ER-mitochondrial communication as a result of increased contacts between the two organelles. As such, decreased contacts between ER and mitochondria will have an opposite effect on MAM-related functions. Cholesterol ester and phospholipid synthesis was assayed in MEFs lacking mitofusin-2 (Mfn2), a protein that is required for MAM-mediated ER-mitochondrial interactions (de Brito and Scorrano, 2008). A ~2-fold lower rate of CE synthesis in Mfn2-KO MEFs (Figure 46A), and a ~60-70% decrease in phospholipid synthesis and transport, as compared to WT MEFs was observed (Figure 46B). γ-secretase activity was examined in deficient ER-mitochondrial
communication present in Mfn2-KO cells. The levels of APP and its C-terminal cleavage products C99 and AICD (APP intracellular domain) were examined (Area-Gomez et al., 2009) (see scheme in Figure 46C). There was a ~2-fold reduction in the amount of AICD in Mfn2-KO cells as compared to WT, and a concomitant increase in C99 (Figure 46C). As a control, these 3 proteins were measured in WT and PS-DKO MEFs. No AICD was produced in the DKO cells (Area-Gomez et al, 2009), while C99 accumulated accordingly (Figure 46C). The deficiency in γ-secretase activity in Mfn2-KO cells was not due to a re-localization or mislocalization of presenilins in these cells, as they were still highly enriched in the MAM compartment (Figure 46D). Thus, the deficiency in γ-secretase activity in Mfn2-KO cells shows that ER-mitochondrial communication is required for this functionality.


[00323] In view of the enrichment of presenilins in the MAM and the alterations in MAM function and morphology in PS-deficient cells described here, the results described herein show that MAM is an intracellular LR/DRM in which presenilins negatively regulate the connection of ER with mitochondria, and that upregulated MAM function plays a hitherto unrecognized role in the pathogenesis of AD (Schon and Area-Gomez (2010) J. Alzheimers Dis. , in press).

[00324] In view results showing that MAM is the predominant subcellular locus for presenilins and for γ-secretase activity (Area-Gomez et al., 2009), and the significantly increased function and altered morphology of MAM in PS-deficient and AD cells described herein, presenilins act as negative regulators of the connections between ER and...
mitochondria (as opposed to the role of MFN2 in positively regulating these contacts (de Brito and Scorrano, 2008)). Moreover, a corollary of these findings is that many of the morphological and biochemical phenotypes associated with AD are can be consequences of this increased crosstalk.

Apart from the lack of appropriate markers to detect MAM, one of the reasons that MAM was overlooked as a DRM, and as the region in which PSs and γ-secretase activity are enriched, can be that most methods used to isolate rafts do not separate PM from intracellular membranes (Macdonald and Pike, 2005); thus, intracellular DRMs like MAM will be co-isolated with PM rafts during the subfractionation process, obscuring the localization of PSs and γ-secretase activity at these ER-mitochondria connections. Furthermore, the fact that MAM is a DRM can explain the lack of consensus regarding the subcellular localization of presenilins, as detecting proteins embedded in DRMs can be technically challenging (Schon and Area-Gomez, 2010). It can also explain why MAM markers such as VDAC (Szabadkai et al., 2006) and calnexin (Myhill et al., 2008) were found in some studies of PM rafts (Foster et al., 2003; Zheng et al., 2009) and why Aβ was reported to be present in mitochondria (Lustbader et al., 2004). Likewise, crude mitochondrial preparations contain MAM that can lead one into thinking that mitochondria contain bona fide lipid rafts (Sorice et al., 2009), when in fact they do not (Zheng et al., 2009). Presenilins, APP, Aβ, and γ-secretase activity are enriched in LRs/DRMs (Urano et al., 2005), and APP processing to produce Aβ depends on lipid rafts (Ehehalt et al., 2003). It had always been assumed that rafts are located at the PM (Lajoie and Nabi). The data described herein show that APP processing to produce Aβ occurs not at the PM lipid rafts, but intracellularly, in the MAM (Hayashi and Fujimoto, 2010). Intracellular rafts have been reported in the ER or mitochondria (Browman et al., 2006); the results described herein show these LR/DRMs are, in fact, MAM (Hayashi and Fujimoto, 2010).

As a place for integration of key cellular functions and signaling (Hayashi et al., 2009), MAM requires a more rigid liquid-ordered phase to recruit, orient, regulate, and limit the lateral mobility of the membrane proteins within this region, so as to promote the crosstalk of mitochondria and ER. Taking this into consideration, alterations of MAM structure and composition, and its nature as a lipid raft, may affect the cellular functions localized within it. Loss of presenilin function induces the continuous activation of these lipid rafts, and that pathological presenilin mutations can similarly alter its lipid organization in such a manner that MAM function and APP cleavage are affected.
Increased ER-mitochondrial communication in AD patient cells is relevant to the pathogenesis of the disease, because many of the apparently unrelated cellular functions that are misregulated in AD can be ascribed to increased ER-mitochondrial communication. First, it has long been known that AD patients have elevated cholesterol (Stefani and Liguri, 2009), elevated ACAT levels (Pani et al., 2009), and neuronal deposition of lipid droplets (Gomez-Ramos and Asuncion Moran, 2007). In addition, MAM plays a role in lipid droplet formation (Walther and Farese, 2009) and that ACAT activity is required for Aβ production (Puglielli et al., 2004). While it is unclear how mutated presenilins affect Aβ production, one possibility is that altered cholesterol and lipid composition changes the orientation of APP within the MAM membrane and its cleavage by γ-secretase, and hence, the generation of total Aβ and the Aβ42:Aβ40 ratio (Uemura et al., 2011). A connection between MAM and plaque formation is also supported by the fact that extracellular plaques have an intracellular origin, and that this process is mediated by sphingomyelin and GM1 gangliosides (Yuyama and Yanagisawa, 2010), both of which are components of MAM and which play a significant role in its regulation (Sano et al., 2009). In addition, the finding of increased phospholipid synthesis in PS-mutant cells, indicative of increased cross-talk between ER and mitochondria (Voelker, 2005), is consistent with the reported aberrations in phospholipid profiles in AD patients (Pettegrew et al., 2001).

The alterations in MAM likely affect other cellular functions as well. One of the most critical of these is calcium homeostasis (Csordas et al., 2010), as MAM is highly enriched in sarcoendoplasmic reticulum calcium-ATPase (de Meis et al., 2010), the sigma-1 type non-opioid receptor (Hayashi and Fujimoto, 2010), ryanodine receptors (RyRs) (Garcia-Perez et al., 2008), and inositol-1,4,5-trisphosphate receptors (IP3Rs) (Hayashi et al., 2009). Thus, increased ER mitochondrial communication in AD could explain the altered intracellular Ca2+ trafficking via RyRs (Stutzmann et al., 2006) and IP3Rs (Leissring et al., 1999; Zampese et al., 2011), leading to the aberrant calcium homeostasis found in patients (Bezprozvanny and Mattson, 2008).

Mitochondrial dysfunction in AD has been reported extensively (Su et al., 2010), but there is no clear evidence as to whether it is cause or effect. Altered connections between MAM and mitochondria almost certainly would affect mitochondrial dynamics (e.g. shape, distribution, and movement) and function (e.g. oxidative energy metabolism, calcium buffering capacity, and free radical production), and thus may be the underlying cause of the reported aberrant mitochondrial phenotypes seen in AD (Su et al., 2010).
**[00330]** Finally, the results described herein have implications for understanding the genetics of AD (Hollingworth et al., 2011; Naj et al., 2011). Most presenilin mutations causing FAD are dominant and are presumed to cause a gain of function. However, the increased MAM function in PS-KD and -KO cells reported here imply that the effects of many pathogenic PS1 mutations are more likely to be due to loss of function or gain of negative function (McMillan et al., 2000; Shen and Kelleher, 2007). Moreover, the remarkable similarity in MAM-related phenotypes of cells from PS-mutant FAD patients to those from SAD patients in which PS structure (but perhaps not expression (McMillan et al., 2000)) is presumably normal implies that there likely is a common pathogenic mechanism - namely increased ER-mitochondrial communication - underlying both the familial and sporadic forms of the disease.

**[00331]** The increased MAM function and ER-mitochondria crosstalk found in all PS mutant cells analyzed can explain some of the features (Schon and Area-Gomez, 2010) and early events in AD, such as elevated serum cholesterol levels, mitochondrial dysfunction, oxidative stress, and calcium deregulation, which are all upstream the appearance of plaques and tangles (Pratico and Delanty, 2000) and which are detected routinely in AD patients. As such, upregulated MAM function could play a hitherto unrecognized and critical role in the pathogenesis of AD (Schon and Area-Gomez, 2010) that can further contribute an understanding of this devastating disease.

**[00332]** The following methods can be used in connection with the embodiments of the invention.

**[00333]** **Subcellular fractionation and Western blotting**

**[00334]** Purification of ER, MAM, and mitochondria was performed and analyzed as described herein.

**[00335]** **Lipid droplet staining**

**[00336]** Staining of lipid droplets was performed using HCS LipidTox™ Deep Green neutral lipid stain according to manufacturer instructions. Lipid droplet staining was quantified using ImageJ. The different values represent the product of the intensity and the area covered by the fluorescent signal above background in every cell examined. For lipid droplet staining by Oil Red O, we first prepared the Oil O red stock solution (35% of Oil O Red in isopropanol), stirring the mix for 12 h and filtering it before use. Cultured cells were fixed in 10% formalin in PBS for at least 1 h and washed twice in ddH2O. The plates were
incubated in 60% isopropanol for 5 min at RT, the alcohol discarded, and the cells were allowed to dry completely at RT. One ml of a filtered Oil Red O working solution (60% of Oil O red stock solution in H2O) was added to the cells at RT for 10 min. The Oil Red O solution was removed and samples were immediately washed in ddH2O before acquiring images under the microscope for analysis.

[00337] Isolation of lipid rafts

[00338] To identify detergent-resistant domains, samples were resuspended in 400 µl of isolation buffer (IB: 250 mM mannitol, 5 mM HEPES pH 7.4, and 0.5 mM EGTA) containing 1% Triton X-100 (TX100) and incubated at 4°C with rotation for 1 h. Samples were adjusted to 80% sucrose, placed at the bottom of a 5-30% sucrose gradient, and centrifuged at 250,000 x g for 18 h. After fractionation, equal volumes of each fraction were loaded on an SDS-PAGE gel and analyzed by Western blot.

[00339] Measurement of cholesterol and cholesteryl esters

[00340] Quantification of total cholesterol and cholesteryl esters was performed using the Cholesterol/Cholesteryl Ester Quantitation kit).

[00341] Analysis of phospholipid synthesis in cultured cells

[00342] Cells were incubated for 2 h with serum free medium to ensure removal of exogenous lipids. The medium was then replaced with MEM containing 2.5 µCi/ml of 3H-serine for the indicated periods of time. The cells were washed and collected in DPBS, pelleted at 2500 x g for 5 min at 4°C, and resuspended in 0.5 ml water, removing a small aliquot for protein quantification. Lipid extraction was done following the Folch method. Briefly, 3 volumes of chloroform:methanol 2:1 were added to the samples and vortexed. After centrifugation at 8000 x g for 5 min, the organic phase was washed twice with 2 volumes of methanol/water 1:1, and the organic phase was blown to dryness under nitrogen. Dried lipids were resuspended in 60 µl of chloroform:methanol 2:1 and applied to a TLC plate. Phospholipids were separated using two solvents composed of petroleum ether-diethyl ether/acetic acid 84:15:1 v/v, and chloroform/methanol/acetic acid/water 60:50:1:4 v/v. Development was performed by exposure of the plate to iodine vapor. The spots corresponding to the relevant phospholipids were scraped and counted in a scintillation counter (Packard Tri-Carb 2900TR).

[00343] Analysis of phospholipid synthesis in subcellular fractions
Crude mitochondrial (CM) fractions were isolated from WT, PS1-KO, PS2-KO and DKO MEFs as described herein. Two hundred µg were incubated in a final volume of 200 µl of phospholipid synthesis buffer (10 mM CaCl2, 25 mM HEPES pH 7.4 and 3 µCi/µl 3H-Ser) for 30 min at 37°C. The reaction was stopped by addition of 3 volumes of chloroform/methanol 2:1. Lipid extraction and TLC analysis was performed as described above.

**Assay of ACAT activity**

To measure ACAT activity in vivo, whole cells were incubated in serum-free medium for 2 h to remove all exogenous lipids. After that, 2.5 µCi/µl of 3H-cholesterol was added to FBS-free DMEM containing 2% FFA-BSA, allowed to equilibrate for at least 30 min at 37°C, and the radiolabeled medium was added to the cells for the indicated periods of time. Cells were then washed and collected in DPBS, removing a small aliquot for protein quantification. Lipids were extracted as described above and samples were analyzed by TLC along with an unlabeled cholesteryl ester standard. A mixture of chloroform/methanol/acetic acid 190:9:1 was used as solvent. Iodine stains corresponding to cholesteryl ester bands were scraped and counted.

To measure ACAT activity in vitro, subcellular fractions were isolated from different tissues, as described herein. Immediately after fractionation, 100 µg of each sample were assayed by mixing it with Buffer A (20 mM Tris-HCl, 1 mM EDTA; pH 7.7) containing 10 mg/ml FAFBSA and 50 µg/ml cholesterol. After 5 min incubation at 37°C, the reaction was started by adding 50 µl Buffer A containing 2 mg/ml FAF-BSA and 3H-oleoyl-CoA, and incubating at 37°C for 20 min. The reaction was stopped by adding chloroform:methanol (1:1) containing 15 µg cholesteryl oleate as a carrier. Known amounts (2-5 µCi) of 3H-cholesterol were added as an internal standard. Lipids extraction and TLC analysis were as above.

**Transmission electron microscopy**

Cells were fixed with 2.5% glutaraldehyde in 0.1 M Sorenson's phosphate buffer (pH 7.2) for at least 1 h. Cells were then postfixed for 1 h with 1% OsO4 in Sorenson's buffer. Staining was performed using 1% tannic acid. After dehydration, cells were embedded in a mixture of Lx-1 12 (Ladd Research Industries) and Embed-812 (EMS, Fort Washington, PA). Thin sections, cut on an MT-7000 ultramicrotome, were stained with uranyl acetate and lead citrate, and examined in a JEOL JEM-1200 EXII electron microscope.
microscope. Pictures were taken on an ORCA-HR digital camera (Hamamatsu) and recorded with an AMT Image Capture Engine.

[00350] Cinnamycin sensitivity assays

[00351] To measure cinnamycin binding (Emoto et al. (1999) Proc. Natl. Acad. Sci. USA 96:12400-12405), cells are incubated with 1251-labeled streptavidin complexed with cinnamycin (Ro 09-0198) peptide complex (125I-SA-Cin; 50,000 cpm/ml; Sigma) for 1h at 39.5°C. The radioactivities of 125I SA-Cin bound to the cells is analyzed by bioimage analyzer. To measure cell viability (Choung et al. (1988) Biochim. Biophys. Acta 940:171-179), cells are incubated with varying concentrations of cinnamycin (0.01 - 100 mM) from 1 - 30 min at 37°C in order to determine the MIC and/or time to kill 50% of the cells (LC50; ~1 mM at ~2 min for human erythrocytes). Viability will be measured by "live/dead" assay (Molecular Probes).

[00352] Knockdown of PS1 expression

[00353] Small hairpin (sh) RNA oligonucleotides M2 @ nt 179-197 in NM_008943: (gacaggtggtggacaaga) and mismatch control shRNAs (Medema RH (2004) Biochem. J. 380:593-603) M3 (gacaggaggaggaacaaga, mismatches underlined) were inserted into pSUPER-Retro vector pSR (OligoEngine). In some experiments the puromycin-resistance cassette was replaced with a blasticidin-resistance cassette, generating pSR-Blast to allow for "double transduction" using two different selection markers to increase shRNA expression. Viral supernatants (3 ml) from plasmid-transfected Amphotrophic Phoenix phi-X-A packaging cells (Kinsella TM, Nolan GP (1996) Hum. Gene Ther. 7:1405-1413) supplemented with polybrene were added to MEFs, seeded 1 day prior to infection at 100,000/well in 6- well culture plates, and infection was allowed for 24 hours. Cells were selected in medium containing puromycin, blasticidin, or both antibiotics, for 14 days.

[00354] Analysis of the role of PS1 in mitochondrial bioenergetics

[00355] The results described herein indicate that PS1-mutant cells have altered mitochondrial function (e.g. 02 consumption; ATP synthesis; free radical production), consistent with data already in the literature (e.g. Hirai et al. (2001) J. Neurosci. 21:3017-3023), but the degree and extent of such dysfunction requires further exploration.

[00356] The bioenergetics - respiratory chain activity, oxygen consumption, ATP synthesis, membrane potential, and ROS production under different metabolic conditions -
will be examined in a wider range of PS 1-mutant cells (e.g. patient fibroblasts, mouse KD neuroblastoma cells, KO, and dKO cells), and where available, in mitochondria isolated from brains of WT and PS 1-mutant mice. The mitochondrial ROS production will be examined as H202 emission fluorimetrically (see Andreyev et al. (2005) Biochemistry (Moscow) 70:200-214 for details). Using Amplex Red, H202 emission rates can be measured with NAD+ and FAD-linked respiratory substrates such as pyruvate, malate, and succinate, and compared with rates of O2 consumption and the membrane potential of isolated mitochondria. To measure H202 scavenging capacity, two protocols can be used (as described herein) that employ physiologically realistic concentrations of H202 (up to 4 μM) and which measure two characteristics of the ROS-scavenging system: tolerance to acute H202 insult and ability to withstand a continuous H202 challenge. The H202 data will be correlated with a visual readout of ROS, using MitoSox.

[00357] The rates of ROS production obtained by these protocols in the absence of respiratory chain inhibitors also depend upon the magnitude of the membrane potential in mitochondria. Therefore, upon detecting any differences in mitochondrial ROS production between genetically modified mice and their littermates, the amplitude of their membrane potential will be measured under the identical conditions.


[00359] H2O2 production

[00360] H2O2 production is measured with a horseradish peroxidase/Amplex Red detection system. Mitochondria are resuspended in standard incubation buffer (SIB) supplemented with either pyruvate and malate or with succinate and with 40U/ml superoxide dismutase (Starkov et al. (2002) J. Neurochem. 83:220-228; Starkov et al. (2004) J. Neurosci. 24:7779-7788; Smaili et al. (2003) Brazil. J. Med. Biol. Res. 36:183-190). Calibration is performed by infusion of known amounts of H2O2 with a microdialysis pump. H2O2 scavenging capacity of mitochondria. A robust microtiter plate protocol that is quick, reproducible, and requires no more than 2-5 µg of mitochondria per assay can be used. The incubation buffer (IB) is composed of SIB and desired oxidative substrates. Two sets of microtiter plate wells are loaded with IB supplemented with variable H2O2 (0 - 800 pmol H2O2) per well. The reaction is triggered by adding mitochondria suspended in IB free of H2O2 to one set of wells; the second set is loaded with an equivalent volume of IB free of H2O2. After 5 min incubation at 370C, both sets are loaded with H2O2 detection mixture composed of 20 U/ml horseradish peroxidase and 10 µM Amplex Red in IB, and the fluorescence intensity of formed resorufin is measured with multifunction plate reader (SpectraMax M5, Molecular Devices, USA). Residual H2O2 is calculated from a calibration curve obtained by measuring the fluorescence of a standard solution of resorufin, which is the reaction product of Amplex Red with H2O2/horseradish peroxidase. Scavenging capacity = difference in H2O2 between wells ± mitochondria. Membrane potential. Besides using TMRM/TMRE to visualize mitochondrial membrane potential in cells, membrane potential will be quantitated using the membrane potential-sensitive dye safranin O, added at 20:1 (mM dye:mg protein) Feldkamp et al. (2005) Am. J. Physiol. Renal Physiol. 288:F1092-F1102, either spectrophotometrically or with a TPP+ selective electrode (Capell et al. (1997) J. Neurochem. 69:2432-2440).

[00361] Isolation and purification of subcellular fractions
Purification of ER, ER-MAM, and mitochondria was performed essentially as described (Stone SJ, Vance JE (2000) J. Biol. Chem. 275:34534-34540; Vance JE (1990) J. Biol. Chem. 265:7248-7256). Cells and tissues were washed and immersed in isolation buffer (250 mM mannitol, 5 mM HEPES pH 7.4, 0.5 mM EGTA, and 0.1% BSA). Tissues were homogenized gently by four strokes in a loose Potter-Elvehjem grinder (Kontes). The homogenate was centrifuged for 5 min at 600 g to remove cells debris and nuclei. The supernatant was subjected to centrifugation for 15 min at 10,500 g, yielding two fractions: the supernatant, containing the ER/microsomal fraction, and the pellet, containing the crude mitochondrial fraction. The supernatant was subjected to centrifugation for 1 h at 100,000 g to pellet the ER/microsomal fraction. The crude mitochondrial fraction was layered on top of a 30% Percoll gradient and centrifuged for 30 min at 95,000 g in a Beckman Coulter Ultracentrifuge (Vance et al. (1997) Biochim. Biophys. Acta 1348:142-150). Two clear bands were visible in the gradient, an upper (lower-density) band containing the ER-MAM fraction and a lower (higher density) band containing mitochondria free of ER. Both fractions were recovered and washed with isolation buffer and pelleted at 10,500 g for 15 min, twice, to eliminate the Percoll.

To obtain the plasma membrane (PM) fraction, tissues were homogenized in STM 0.25 buffer (0.25 M sucrose, 10 mM Tris-Cl pH 7.4, 1.0 mM MgCl2), using a loose-fitting Potter-Elvehjem grinder (10 strokes). Homogenates were centrifuged for 5 min at 260 g and the supernatant was kept on ice. The pellet, containing nuclei and cell debris, was resuspended in half the volume of the same buffer and homogenized with three strokes on the same loose grinder and pelleted again for 5 min at 260 g. Both supernatants were combined and centrifuged for 10 min at 1,500 g. The pellet, containing the PM, was resuspended in twice the volume of STM 0.25 used initially and was further homogenized by three strokes, but using a tight-fitting grinder. The homogenate was diluted by adding an equal volume of STM 2 buffer (2 M sucrose, 10 mM Tris-Cl pH 7.4, 1.0 mM MgCl2), and centrifuged for 1 h at 113,000 g. The resulting low-density thin layer located near the top of the gradient, enriched in PM, was resuspended in 0.5-1 volume of STM 0.25 buffer.

Cinnamycin Binding Assay

Binding assay (modified from Emoto et al. (1999) Proc. Natl. Acad. Sci. USA 96:12400). Wild-type or PSI-mutant cells are seeded into 100-mm diameter dishes at 5 x 10^3 cells per dish and cultivated at 33°C for 20 days. The cell colonies are replicated onto polyester disks. The polyester discs are incubated for 24 h in growth medium at 39.5°C,
washed twice with F-12 medium, and then incubated with $^{125}$I-labeled streptavidin complexed with cinnamycin (Ro 09-0198) peptide complex ($^{125}$I-SA-Cin; 50,000 cpm/ml) for 1h at 39.5°C. The radioactivities of $^{125}$I-SA-Cin bound to the colonies is analyzed by bioimage analyzer. Mutant cells will exhibit a lower binding activity than control cells.

[00366] Cinnamycin Viability Assay

[00367] Viability assay (modified from Choung et al. (1988) Biochem. Biophys. Acta 940:171). Normal fibroblasts are incubated with varying concentrations of cinnamycin (0.01 - 100 mM in log dilutions for times ranges from 1 - 30 min at 37°C in order to determine the normal concentration and/or time to kill 50% and 100% of the cells (LC$_{50}$ and LC$_{100}$; the LC$_{50}$ for normal human erythrocytes is ~ 1 mM with an incubation time of ~2 min). Viability can be measured many ways. In one embodiment, cell viability can be measured with a "live/dead" assay (Molecular Probes) that stains living cells as green and dead cells red. PS1-mutant cells are treated under the same conditions to determine if the are resistant to cinnamycin. In another embodiment, the viability of cells in the presence of cinnamycin can be determined by measuring the LC$_{50}$ and LC$_{100}$ for PS1-mutant cells compared to control cells.

[00368] Culturing of explanted primary mouse neurons

[00369] Mice are sacrificed in C02 and soaked in 80% ethanol for 10 min. Fetuses are removed (E15 mouse embryos) and kept in PBS on ice. After removal of the meninges, the cortex is dissected, and washed with Hank's balanced salt solution (HBSS). Cortical neurons are released from tissue by trypsin treatment, followed by trituration, and plated on polylysine coated culture dishes at a density of ~106 cells/35-mm dish (Friedman et al, 1993) Differential actions of neurotrophins in the locus coeruleus and basal forebrain. Exp. Neurol. 119:72-78). Prior to experiments, cells are maintained for 4-5 days in serum-free medium and 0.5 mM 1-glutamine (Rideout HJ, Stefanis L (2002) Proteasomal inhibition-induced inclusion formation and death in cortical neurons require transcription and ubiquitination. Mol. Cell. Neurosci. 21:223-238) to yield a relatively pure culture of neurons. To ensure that this is the case, immunostaining for a-internexin, an intermediate filament protein expressed by differentiated postmitotic neurons of the developing CNS, but not by neuroblasts or cells of the glial lineage, can be performed (Fliegner et al., 1994) Expression of the gene for the neuronal intermediate filament protein a-internexin coincides with the

Subcellular fractionation

Purification of ER, ER-MAM, and mitochondria was performed essentially as described (Stone and Vance, J. Biol. Chem. 275,34534 (2000); Vance, Biol Chem. 265, 7248 (1990)). Cells and tissues were washed and immersed in isolation buffer (250 mM mannitol, 5 mM HEPES pH 7.4, 0.5 x EGTA, and 0.1% BSA). Tissues were homogenized gently by four strokes in a loose Potter-Elvehjem grinder (Kontes). The homogenate was centrifuged for 5 min at 600 g to remove cells debris and nuclei. The supernatant was subjected to centrifugation for 15 min at 10,500 g, yielding two fractions: the supernatant, containing the ER/microsomal fraction, and the pellet, containing the crude mitochondrial (CM) fraction. The supernatant was subjected to centrifugation for 1 h at 100,000 g to pellet the microsomal fraction. The crude mitochondrial fraction was layered on top of a 30% Percoll gradient and centrifuged for 30 min at 95,000 g in a Beckman Coulter Ultracentrifuge: two clear bands were visible in the gradient, an upper (lower-density) band containing the ER-MAM fraction and a lower (higher density) band containing mitochondria free of ER; both fractions were recovered and washed with isolation buffer and pelleted at 10,500 g for 15 min, twice, to eliminate the Percoll. All fractions were quantitated for total protein content using the Bradford system (BioRad).

To obtain the plasma membrane (PM) fraction, tissues were homogenized in STM 0.25 buffer (0.25 M sucrose, 10 mM Tris-Cl pH 7.4, 1.0 mM MgCl$_2$; 4.5 ml/g tissue), using a loose-fitting Potter-Elvehjem grinder (Kontes) (10 strokes). Homogenates were centrifuged for 5 min at 260 g and the supernatant was kept on ice. The pellet, containing nuclei and cell debris, was resuspended in half the volume of the same buffer and homogenized with three strokes on the same loose grinder and pelleted again for 5 min at 260 g. Both supernatants were combined and centrifuged for 10 min at 1,500 g. The pellet, containing the PM, was resuspended in twice the volume of STM 0.25 used initially and was further homogenized by three strokes, but using a tight-fitting grinder (Kontes). The homogenate was diluted by adding an equal volume of STM 2 buffer (2 M sucrose, 10 mM Tris-Cl pH 7.4, 1.0 mM MgCl$_2$), and centrifuged for 1 h at 113,000 g. The resultant low-density thin layer located near the top of the gradient, enriched in PM, was resuspended in 0.5-1 volume of STM 0.25 buffer (D. E. Vance, C. J. Wakey, Z. Cui, Biochim. Biophys. Acta 1348, 142 (1997).
Purification and analysis of subcellular fractions from mouse liver

Plasma membrane (PM), crude mitochondria (CM), and ER was isolated as described herein (Stone and Vance, J. Biol. Chem. 275,34534 (2000); Vance, Biol. Chem. 265, 7248 (1990)), and fractionated crude mitochondria further by isopycnic centrifugation (Vance, et al, Biochim. Biophys. Acta 1348, 142 (1997)) into a ER-MAM fraction and a purified mitochondrial fraction. Each of these fractions was evaluated by Western blot analysis using antibodies to adherin (CDH2) as a marker for PM, to calnexin (CANX) as a marker for ER, to Golgi matrix protein GM130 (GOLGA2) as a marker for Golgi, to ACATI, G6PC, and PEMT as markers for ER-MAM (and to a lesser extent, ER), and to the NDUFA9 subunit of complex I of the respiratory chain as a marker for mitochondria. The ER-MAM fraction is distinct from ER or purified mitochondrial fractions, specifically the, ER-MAM fraction was enriched for the three ER-MAM markers. These three proteins were significantly less enriched in the ER and mitochondrial fractions compared to ER-MAM. Similarly, marker proteins for the PM, Golgi, ER and mitochondria were selectively depleted from the ER-MAM fraction (Fig. 10).

Purification of ER, ER-MAM, and mitochondria was performed as described (S. J. Stone, J. E. Vance, J Biol. Chem. 275,34534 (2000); J. E. Vance, 3:Biol. Chem. 265,7248 (1990)). Cells and tissues were washed and immersed in isolation buffer (250 mM mannitol, 5 mM HEPES pH 7.4, 0.5 mM EGTA, and 0.1% BSA). Tissues were homogenized gently by four strokes in a loose Potter-Elvehjem grinder (Kontes). The homogenate was centrifuged for 5 min at 600 g to remove cells debris and nuclei. The supernatant was subjected to centrifugation for 15 min at 10,500 g, yielding two fractions: the supernatant, containing the ER/microsomal fraction, and the pellet, containing the crude mitochondrial fraction. The supernatant was subjected to centrifugation for 1 h at 100,000 g to pellet the ER/microsomal fraction. The crude mitochondrial fraction was layered on top of a 30% Percoll gradient and centrifuged for 30 min at 95,000 g in a Beckman Coulter ultracentrifuge. Two clear bands were visible in the gradient, an upper (lower-density) band containing the ER-MAM fraction and a lower (higher density) band containing mitochondria free of ER. Both fractions were recovered and washed with isolation buffer and pelleted at 10,500 g for 15 min, twice, to eliminate the Percoll. All fractions were quantitated for total protein content using the Bradford system (BioRad).

To obtain the plasma membrane (PM) fraction, tissues were homogenized in STM 0.25 buffer (0.25 M sucrose, 10 mM TrisCl pH 7.4, 1.0 mM MgCl₂; 4.5 ml/g tissue),
using a loose-fitting Potter-Elvehjem grinder (Kontes) (10 strokes). Homogenates were centrifuged for 5 min at 260 g and the supernatant was kept on ice. The pellet, containing nuclei and cell debris, was resuspended in half the volume of the same buffer and homogenized with three strokes on the same loose grinder and pelleted again for 5 min at 260 g. Both supernatants were combined and centrifuged for 10 min at 1,500 g. The pellet, containing the PM, was resuspended in twice the volume of STM 0.25 used initially and was further homogenized by three strokes, but using a tight-fitting grinder (Kontes). The homogenate was diluted by adding an equal volume of STM 2 buffer (2 M sucrose, 10 mM Tris-Cl pH 7.4, 1.0 mM MgCl₂), and centrifuged for 1 h at 113,000 g. The resultant low-density thin layer located near the top of the gradient, enriched in PM, was resuspended in 0.5-1 volume of STM 0.25 buffer.

[00377] Other methods for isolating ER-MAM are also known to those skilled in the art. As one non-limiting example, ER-MAM fractions can be obtained by immersing a biological sample (e.g. tissues or cells) in an ice-cold isolation medium (250 mM mannitol, 5 mM HEPES, pH 7.4, 0.5 mM EGTA, and 0.1% bovine serum albumin). If the sample is a tissue, it can be minced with scissors and homogenized gently by four strokes in a Potter-Elvehjem motor driven homogenizer. The homogenate can then centrifuged twice at 600 X g for 5 min to remove large debris and nuclei. The supernatant is centrifuged for 10 min at 10,300 x g to pellet the crude mitochondria. Microsomes can be obtained by centrifugation of the resultant supernatant at 100,000 x gmax for 1 hour in a Beckman Ti-70 rotor. For further purification of mitochondria, the crude mitochondrial pellet can be suspended by hand homogenization in approximately 4 ml of isolation medium, and the suspension can be layered on top of 20 ml of medium containing 225 mM mannitol, 25 mM HEPES, pH 7.4, 1 mM EGTA, 0.1% bovine serum albumin, and 30% (v/v) Percoll, in each of four 30-ml polycarbonate ultracentrifuge tubes. The tubes can then be centrifuged for 30 min at 95,000 x gmax, after which a dense band, containing purified mitochondria, can be recovered from approximately 2/3 down the tube. The mitochondria are removed with a Pasteur pipette, diluted with isolation medium, and washed twice by centrifugation at 6,300 x gm for 10 min to remove the Percoll. The final pellet is resuspended in isolation medium and can be stored at -70 °C. ER-MAM can be isolated from the Percoll gradient from the band immediately above the mitochondria, by centrifugation first at 6,300 gmax for 10 min then further centrifugation of the supernatant at 100,000 x gmax, for 1 h in a Beckman Ti-70 rotor. The pellet of ER-MAM, can be resuspended in approximately 0.5 ml of buffer containing 0.25 M
sucrose, 10 mM Tris-HCl, pH 7.4, and 0.1 mM phenylmethylsulfonyl fluoride, and stored at -70 °C.

[00378] For subfractionation of mitochondria into inner and outer membranes, the pure mitochondrial pellet can be suspended in buffer (20mg/ml) containing 70 mM sucrose, 200 mM mannitol, and 2 mM HEPES, pH 7.4. The mitochondria (2.5 mg) can be mixed gently with 125 µl of 0.6% digitonin solution made in the above buffer and incubated on ice for 15 min. The mixture can be diluted with the above buffer containing 50 mg of bovine serum albumin/100 ml, then centrifuged for 10 min at 12000 x g_{max}. The supernatant is enriched in mitochondrial outer membranes, and the pellet is enriched in inner membranes. Methods for isolating Golgi, plasma membrane, and rough and smooth endoplasmic reticulum fractions are known to one skilled in the art (for example see Croze, E. M., and Morre, D. J. (1984) J. Cell. Physiol. 119, 46-52. Dennis, E. A., and Kennedy, E. P. (1970) J. Lipid Res. 11, 394-403).

[00379] Methods for isolating crude mitochondria are known to those skilled in the art (for example see, Vance, 1990 or Croze and Morre, 1984). ER-MAM and purified mitochondria can be separated on a self-forming 30% Percoll gradient (Vance, 1990; Hovius et al., 1990). Golgi membranes and two ER fractions (ERI and ERII) can be isolated (Croze and Morre, 1984). ERI can be obtained from the final sucrose gradient at the interface between sucrose solutions of 1.5 and 2.0M, whereas ERII can be isolated from the interface between sucrose solutions of 1.5 and 1.3 M. ERI is enriched in rough ER membranes, and ERII in smooth ER membranes (Croze and Morre, 1984).

[00380] Various methods to examine the activity of biomarkers of subcellular fractions have been described in the art. The ER marker enzymes NADPH:cytochrome c reductase and glucose-6-phosphate phosphatase can be assayed by established procedures (Vance and Vance, 1988). Enzymatic activity for UDP:N-acetylglucosamine-1-phosphotransferase (Rusiol et al., 1993), UDP:N-galactose-acetylglucosaminegalactosyltransferase (Rusiol et al., 1993b), and cytochrome C oxidase (Vance and Vance, 1988) can also be measured by methods known in the art.

[00381] Mitochondrial Superoxide Stress Fluorescence Assay ("MitoSox")

[00382] Mitosox Red (Molecular Probes) is live-cell permeant and that is selectively targeted to mitochondria. Once inside the mitochondria, the reagent is oxidized by superoxide and binds to nucleic acids, resulting in a red fluorescence. Normal fibroblasts do
not stain with MitoSox, whereas PS1-mutant cells. Staining of mitochondria indicates superoxide radical production. A more general assay that detects many forms of reactive oxygen species (ROS) (e.g. superoxide, hydrogen peroxide, singlet oxygen, and peroxynitrite) can also be used. One technique is to use "Image-iT Live" assay (Molecular Probes), which is based on 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA). Carboxy-H2DCFDA is a fluorogenic marker for ROS. Non-fluorescent carboxy-H2DCFDA permeates live cells and is deacetylated by nonspecific intracellular esterases. In the presence of ROS, which are produced throughout the cell (particularly during oxidative stress), the reduced fluorescein compound is oxidized and emits green fluorescence.

[00383] Immunofluorescence

[00384] To detect mitochondria, living cells were labeled with 1 nM MitoTracker Red CMXRos (MTred; Invitrogen) in DMEM for 20 min at 37°C. The cells were fixed after washing the cells in DMEM twice for 10 min, as described herein.

[00385] For immunolocalization, cells were fixed and permeabilized using three different methods: (1) fixation in 4% paraformaldehyde (PF) for 30 min at RT and permeabilization in either 0.1% or 0.4% Triton X-100 (TX100) for 15 min at RT; (2) fixation in 4% PF for 30 min at RT and permeabilization in chilled methanol for 20 min at -20°C; and (3) fixation and permeabilization in chilled methanol for 20 min at -20°C. The fixed cells were then washed twice for 5 min in phosphate-buffered saline (PBS), and incubated in blocking solution (2.5% normal goat serum [NGS], 1% bovine serum albumin [BSA], and 0.1% TWEEN-20 in 1x PBS) in a humid chamber. Incubation with primary antibodies was performed at room temperature (RT) as recommended. Secondary antibodies were used according to the manufacturers’ instructions. For simultaneous detection of PEMT and PS1, PEMT was detected by treating the cells first with rabbit anti-PEMT, then with mouse anti-rabbit IgG ("bridge" antibody), and finally with goat anti-mouse IgG conjugated to Alexa Fluor 594 (red) (Invitrogen), while PS1 was detected by treating cells with rabbit anti-PS1 and then with goat anti-rabbit IgG conjugated to Alexa Fluor 488 (green). Detection was also performed reversely (i.e. PEMT using goat anti-rabbit IgG and PS1 using mouse anti-rabbit followed by goat anti-mouse). For detection of cdnexin, secondary antibodies conjugated to Alexa Fluor 350 (blue) were used.
[00386] Cells were imaged on an Olympus 1x70 inverted microscope. Red, green, and blue images were captured sequentially using a SPOT RT digital camera and merged using SPOT RT software (New York/New Jersey Scientific, Inc.). Confocal microscopy was performed with a Zeiss LSM510 microscope using a 63x Plan-Neofluor, 1-25 NA objective lens. The pinhole was set to give an optical section of 1.1 μm. Excitation was at 488 nm (for green), 543 nm (for red), and 350 nm (for blue). To quantitate the localization of mitochondria, a region that extended from portions of the nuclear envelope to points midway to the plasma membrane was defined, and the amount of MitoTracker Red signal outside this region was measured. Since organelles are more sparse in the extremities, the dynamic range of signal was higher, and presumably more linear, than signal from the perinuclear region. Specifically, a z-series. of images covering the total cell thickness was collected with a Zeiss LSM510 microscope using a Plan-Neofluor, 0.9 NA objective lens. The pinhole was set to give an optical section of 1.4 μm- The interval between z slices was set to 1.4 μm to give non-overlapping sections. Excitation was at 488 nm (for green) and 543 nm (for red). Z sections were projected onto a single image, and an area between the nucleus and the cell periphery, as determined by microtubule staining, was outlined. In that area, the midpoint between the nucleus and the farthest point at the cell periphery was determined at various positions around the nucleus. Using the midpoints, the outlined area was divided into two parts, one proximal (A) and one distal (B) to the nucleus. Mean grayness values were recorded for the proximal and distal parts. For quantification of mitochondria in the outer edges of cell, the grayness value for the distal part was divided by the grayness value for the total area (proximal + distal). Calculation of grayness value for the total area was ((graynessA x areaA + (graynessB x areaB)) / (areaA + areaB)).

[00387] Mitochondrial Distribution Assay

[00388] Mitochondria in many PS1-mutant fibroblasts are more concentrated around the nucleus than are mitochondria in controls, with fewer mitochondria at the extremities of PS1. This effect can be quantitated by measuring the intensity of the orange signal in the extremities of Mitotracker-stained cells. Measurements are performed by projecting confocal imaging z sections into a single image. An area between the nucleus and the cell periphery, as determined by microtubule staining. The area is outlined, and the midpoint between the nucleus and the farthest point at the cell periphery is determined. Using the midpoint, the outlined area is then divided into two parts: regions proximal (A) and distal (B) to the nucleus. Mean grayness values of the MitoTracker stain are recorded for the
proximal and distal parts. For quantification of mitochondria in the outer edges of a cell, the
grayness value for the distal part is divided by the grayness value for the total area (proximal 
+ distal). Calculation of grayness value for the total area = ([Grayness_{A} \times \text{Area}_{A}] + 
[Grayness_{B} \times \text{Area}_{B}])/(\text{Area}_{A} + \text{Area}_{B}). Significantly fewer mitochondria are observed in the 
extremities of PS I-mutant cells as compared to control cells.

[00389] Immunohistochemistry in brain

[00390] Immunohistochemistry in brain can be performed on 10-μ-thick paraffin-
embedded sections using the ABC method or by double-labeling methods with different 
fluorochromes to human mitochondrial proteins (e.g. COX II, ND1, the iron-sulfur [FeS] 
techniques (histochemical, immunohistochemical, and \textit{in situ} hybridization staining methods) 
to visualize mitochondria. \textit{Methods Cell Biol.} 65:31 1-332). Monoclonal anti-MAP2, a 
perikaryon and dendritic marker, and monoclonal anti-MAP5, a marker for neuronal axons 
can be used for neuronal probes. Additional sections can be stained with H-E for 
conventional microscopic study, with thioflavine S for localization of amyloid deposits, and 
with a modified Bielschowsky silver stain for evaluation of plaques and neurofibrillary 
tangles.

[00391] Cells and reagents

[00392] Mutant FAD^{PS1}-A246E (AG06840 and AG06848) human fibroblasts were 
obtained from the Coriell Institute for Medical Research (Camden, NJ). FAD^{PS1} M146L 
(GG1, GG3, and GG5) and control (GG2, GG4, and GG6) fibroblasts have been described 
elsewhere (R. Sherrington et al., Nature 375,754 (1995)). Normal human fibroblasts (line 
AE) were also used. Other human fibroblast samples were obtained from the University of 
Washington Alzheimer Disease Research Center. Cultured primary rat neurons were obtained 
from Columbia University. Human fibroblasts (line 97) and 3T3 and COS-7 cells were 
available in the laboratory. Mouse embryonic fibroblasts (MEFs) were derived from pups 
with a heterogeneous C57BL161 129Sv background at E 12.5-14.5. Spontaneously-
imortalized MEFs were obtained by passage through the replication bottleneck using a 3T3 
subculture schedule. Cells went into a metabolic crisis between passages 9-12, and recovered 
by passage 16. Retroviral transduction was carried out between passages 20-25. Cells were 
cultured in DMEM medium supplemented with 10% FBS (Invitrogen) and 
penicillin/streptomycin.
[00393] Transfection of mitoDendra

[00394] MitoDendra can be transfected into neurons as described (Ackerley et al., (2000) Glutamate slows axonal transport of neurofilaments in transfected neurons. *J. Cell Biol.* 150:165-176; Nikolic et al. (1996) The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation. *Genes Dev.* 10:816-825). Typically, 10% of the cells are transfected. This provides a sufficient number of cells to allow for multiple measurements. To improve gene expression efficiency and to minimize non-specific toxicity derived from transfection approaches, the mitoDendra construct can be transferred into an adenoviral vector. Neurons can be imaged 36 hr after transfection.

[00395] Antibodies

[00396] The following polyclonal antibodies recognizing different regions of PS1 were used: aa 31-46 (Sigma P4985), aa 450-467 (Sigma W854), aa 303-316 (Calbiochem PC267), and aa 263-407 ("loop" domain; Calbiochem 529592); polyclonal antibodies recognizing aa 32-46 (B 19.2) and aa 310-330 (B32.1) of mouse PS1 were used (W. G. Annaert et al., J Cell Biol. 147,277 (1999)). Antibodies recognizing cadherin (monoclonal; Sigma C 1821 ), calnexin (monoclonal; Chemicon MAB3 1261, fatty acid-CoA ligase 4 (polyclonal; Abgent AP 2536b), glucose-6-phosphatase (polyclonal (A. Eautier-Stein et al, *Nucl. Acids Res.* 31,5238 (2003)), Golgi matrix protein GM130/GOLGA2 (polyclonal ; Calbiochem CB 1008), NDUFA9 (monoclonal; Molecular Probes A2 13441, ACATI (polyclonal; Abeam ab39327), PEMT (polyclonal (Z. Cui, J. E. Vance, M. H. Chen, D. R. Voelker, D. E. Vance, *J Biol. Chem.* 268 16655 (1993)), protein disulfide isomerase (PDI) (monoclonal; Stressgen SPA-891 1, PACS2 (polyclonal M. Kottgen et al, *EMBO J.* 24,705 (2005)), SSR1 (polyclonal (G. Migliaccio, C. V. Nicchitta, G. Blohel, *J. Cell Biol.* 117, 15 (1992)), and tubulin (monoclonal; Sigma T4026) were also used. Goat secondary antibodies (A- 11008, A-l 1012, and A-l 1046) were from Molecular Probes. Mouse monoclonal anti-rabbit "bridge" antibodies (R1008; used at 1:2000) were from Sigma. Secondary HRP-linked mouse (NXA931) and rabbit (NA934V) antibodies were from GE Healthcare Life Sciences.

[00397] Antibodies to APH-1 (ABR PA1-2010), APP (Landman et al, Proc. Natl. Acad. Sci. USA 2006, 103:19524-19529), ATP synthase subunit a (Molecular Probes A21350), FAACL4 (Abgent AP 2536b), Golgi matrix protein GM130/GOLGA2 (Monoclonal BD transduction #610822), IP3R3 (Millipore AB9076), Na,K-ATPase (Abeam ab7671), nicastrin (Covance PRB-364P), PEN2 (Abeam ab62514), and SSRa (Migliaccio et al, J Cell
Mouse monoclonal anti-rabbit "bridge" antibodies were from Sigma (R1008; used at 1:2000).

[00398] **Western blotting**

[00399] Samples were resuspended in Laemmli buffer, heated for 10 min at 60°C, subjected to polyacrylamide gel electrophoresis, transferred to PVDF membranes (BioRad), and probed with antibodies. Immunostaining bands were revealed by chemiluminescence (West Rco ECL Kit, Pierce).

[00400] **Small hairpin (sh) RNA oligonucleotides**

[00401] Small hairpin (sh) RNA oligonucleotides M2 @ nt 179-197 in NM_008943 (gacaggtggtggaacaaga) (SEQ ID NO: 1) and mismatch control shRNAs M3 (gacaggaggagaacaaga; mismatches underlined) (SEQ ID NO: 2) were inserted into pSUPER-Retro vector pSR (OligoEngine). In some experiments, the puromycin-resistance cassette was replaced with a blasticidin resistance cassette (Nhel-Dralll), generating pSR-Blast to allow for "double transduction" using two different selection markers to increase shRNA expression. Viral supernatants (3 ml) from plasmid-transfected Amphotrophic Phoenix ΦNX-A packaging cells (Kinsella, G. P. Nolan, *Hum. Gene Ther.* 7, 1405 (1996)) supplemented with polybrene were added to MEFs, seeded 1 day prior to infection at 100,000/well in 6-well culture plates, and infection was allowed to proceed for 24 hours. Cells were selected in medium containing puromycin, blasticidin, or both antibiotics, for 14 days.

[00402] **Cell transfections**

[00403] The open reading frame from Gentstorm plasmids (Invitrogen) containing human wt and A246E PS1 cDNAs was amplified using flanking PCR primers containing KpnI and XbaI sites at the 5’ and 3’ ends, respectively. The amplification products were inserted into the unique KpnI and XbaI sites of pcDNA3.1 (Invitrogen). Clones were confirmed by DNA sequencing and transfected into COS-7 and 3T3 cells using Lipofectamine 2000 (Invitrogen). After 24-36 h, transfected cells were treated with neomycin to select for stable transformants.

[00404] **In vitro import assay**
Human PS1 was transcribed using a reticulocyte lysate system and imported into isolated mitochondria as described previously (Leuenberger et al, *EMBOJ.* 18, 4816 (1999)).

**Crosslinking**

In order to identify proteins that interact with presenilin, ER-MAM can be isolated and cross-linked with formaldehyde (or with a small panel of crosslinking agents), the cross-linked material can be solubilized with detergent, and then immunoprecipitated with antibodies to presenilin. A number of crosslinking compounds are commercially available, such as SFAD (Pierce, #27719), a bifunctional crosslinking agent that is photoinitiated and is reactive to amino groups and -CH bonds; other reagents contain groups that are reactive to carboxylates and sulfhydryl groups. Different contact times and concentrations of cross-linker can be used in order to reduce over-cross-linking. The immunoprecipitated proteins can then be subjected to tryptic digestion and mass spectrometry for identification. A small panel of these reagents can be used to cover different chemistries of potential targets (e.g., presenilin can react with the amino reactive end of a given cross linker, but the other protein may not present the proper functional group for the other reactive group on the linker).

**Immunocytochemistry to detect PEMT and Presenilin in cells**

The subcellular localization of both PHMT2 and PS1 was sensitive to conditions used for fixation of samples in preparation for immunocytochemistry. Using paraformaldehyde (PF) fixation and permeabilization with Triton X-100 (TX100), PEMT was found to localize to diffuse or punctate structures that did not co-localize with any obvious subcellular compartment (Fig. 11A). However, when cells were treated with cold methanol (MeOH), PEMT co-localized with MTred-stained structures, especially in the perinuclear region (yellow arrowheads in Fig. 11B). Co-localization with MTred was less apparent in more distal regions of the cell (red arrowheads in Fig. 11B). The apparent localization of PEMT with perinuclear mitochondria may be due to the fact that upon dehydration by MeOH, this MAM-associated protein precipitated at or near adjacent mitochondria. An essentially identical result was obtained upon immunolocalization of PS1 in mouse 3T3 cell and human fibroblasts (Fig. 12). Finally, immunostaining of both PEMT and PS1 in the same cells showed a high degree of co-localization (using bath PF/TX100 [Fig. 12A] and MeOH [Fig. 12B] to permeabilize and fix the cells), indicating that PS1 is indeed enriched in the ER-MAM compartment (Fig. 12).
Detection of Mitochondria

Mitochondria were detected after loading the cells with 1 nM MitoTracker Red CMXRos (MTred; Invitrogen) in tissue culture medium (DMEM) for 20 min at 37°C. After washing the cells in medium twice for 10 min, immunolocalization was then performed, using three different methods to fix and permeabilize the cells: (1) fixation in 4% paraformaldehyde (PF) for 30 min at RT and permeabilization in either 0.1 % or 0.4% Triton X-100 (TX100) for 15 min at RT; (2) fixation in 4% PF for 30 min at RT and permeabilization in chilled methanol for 20 min at -20°C; and (3) fixation and permeabilization in chilled methanol for 20 min at -20°C. The fixed cells were then washed twice for 5 min in phosphate-buffered saline (PBS), and incubated in blocking solution (2.5% normal goat serum [NGS], 1% bovine serum albumin [BSA], and 0.1% TWEEN-20 in 1x PBS) in a humid chamber for 1 h at RT. Antibodies were used as recommended. Cells were imaged on an Olympus 1x70 inverted microscope. Red and green images were captured sequentially using a SPOT RT digital camera and merged using SPOT RT software (New York/New Jersey Scientific, Inc.).

For simultaneous detection of PEMT and PS1 (Fig. 13), PEMT was detected by treating the cells first with rabbit anti-PEMT, then with mouse anti-rabbit IgG ("bridge" antibody), and finally with goat anti-mouse IgG conjugated to Alex Fluor 594 (red) (Invitrogen), while PS1 was detected by treating cells with rabbit anti-PS1 and then with goat anti-rabbit IgG conjugated to Alexa Fluor 488 (green). Detection using the reverse procedure (i.e. PEMT using goat anti-rabbit IgG and PS1 using mouse anti-rabbit followed by goat anti-mouse) yielded a similar result.

Immunohistochemistry to Detect Presenilin in Various Cells

The localization of PS1 and MitoTracker Red (MTrred) was examined in other cells, using MTrred to detect mitochondria and immunocytochemistry using antibodies directed against either the N- or C-terminus of PS1 in cells fixed and permeabilized with MeOH (Fig. 14). Co-localization of PS1 was detected with MTrred in mouse 3T3 cells (Fig. 14A) and rat neurons (Fig. 14B): PS1 co-localized with MTrred in the perinuclear region and within the cell body (yellow arrowheads in Figs. 14A and B), but not with mitochondria that were present in processes that extended from the cell body (red arrowheads in Fig. 14A and 14B).
PS1 staining was observed at adherens junctions in the plasma membrane in confluent human 293T (Fig. 14C) and COS-7 cells, also as seen by others (Georgakopoulos et al., Mol. Cell. 4, 893 (1999; Marambaud et al., EMBOJ. 21,1948 (2002)), confirming a known location for PS1 when using MeOH for fixation and permeabilization.

Transfection of Presenilin in COS-7 Cells

Monkey COS-7 cells were transfected stably with a construct expressing either wild-type PS1 or the A246E mutation, and double-stained for MTred and tubulin (Fig. 7) to recapitulate the mitochondrial maldistribution phenotype often seen in FAD<sup>PS1</sup> fibroblasts by expressing mutated PS1. Transfected cells were compared to untransfected cells or to controls expressing empty vector or wt-PS1.

The open reading frame from Genestorm plasmids (Invitrogen) containing human wt and A246E P51 cDNAs was amplified using flanking PCR primers containing KpnI and XbaI sites at the 5' and 3' ends, respectively. The amplification products were inserted into the unique KpnI and XbaI sites of pcDNA3.1 (Invitrogen). Clones were confirmed DNA sequencing and transfected into COS-7 and 3T3 cells using Lipofectamine 2000 (Invitrogen). After 24-36 h, transfected cells were treated with neomycin to select for stable transformants. Cells were stained with MTred (red) and anti-tubulin (green).

γ-Secretase Activity Assays

Endogenous γ-secretase activity was determined by Western blotting to detect the amount of AICD derived from the cleavage of endogenous APP, as described. (Landman et al, Proc. Natl. Acad. Sci. USA 2006, 103:19524-19529). 50 μg of protein from each fraction was incubated in reaction buffer (10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 7.4) for 3 h at 37°C, followed by Western blotting with anti-APP. As a control, the same samples were assayed in the presence of 2 μM compound E ([((2S)-2-[(3,5-difluorophenyl)acetyl]amino)-N-[(3S)-1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-3-yl]propanamide]; Alexis Biochemicals, ALX270-415-C250), a γ-secretase inhibitor (Hansson et al, J. Biol. Chem. 2004, 279:51654-51660). A FRET-based γ-secretase activity assay was used to detect cleavage of an exogenously-added secretase-specific peptide conjugated to two fluorescent reporter molecules (R&D Systems FP003) in serial dilutions of different subcellular fractions. As a control, the same samples were assayed in the presence of 2 μM compound E.
Visualization of PSl using methanol fixation

Cells (80-90% confluent) are stained with MT Red, fixed and permeabilized by adding MeOH (previously frozen in dry ice) for 20 min at -20°C, and washed out with 1x PBS twice. Cells can also be washed, fixed, and permeabilized without staining with MT Red by adding frozen MeOH directly to the culture. Block cells and continue as with a standard immunofluorescence assay.

Example 2: ER-Mitochondrial Interaction in Familial Alzheimer Disease

Clinically, FAD is similar to SAD but has an earlier age of onset. PSl and PS2 are ubiquitously-expressed aspartyl proteases that are about 50-kDa in size. The active forms of PSl and PS2 are N- and C-terminal fragments (NTF and CTF, respectively), which are produced by cleavage of full-length presenilin in its "loop" domain (Zhou S, Zhou H, Walian PJ, Jap BK (2007) Regulation of γ-secretase activity in Alzheimer's disease. *Biochemistry* 46:2553-2563). PSl and PS2 are components of the γ-secretase complex that processes a number of plasma-membrane proteins, including Notch, Jagged and APP. The γ-secretase complex also contains three other structural subunits: APH1, nicastrin, and PEN2 (De Strooper B (2003) Aph-1, Pen-2, and nicastrin with presenilin generate an active γ-secretase complex. *Neuron* 38:9-12).


**[00428]** The ER is the cell's main store of calcium, which is released upon stimulation by input signals such as inositol 1,4,5-triphosphate (IP3) and sphingosine-1 phosphate (Berridge MJ (2002) The endoplasmic reticulum: a multifunctional signaling organelle. *Cell Calcium* 32:235-249), while the main site of calcium uptake is the mitochondrion. The ER and mitochondria are linked not only biochemically but also physically (Csordas et al., (2006) Structural and functional features and significance of the physical linkage between ER and mitochondria. *J. Cell Biol.* 174:915-921; Jousset et al, (2007) STIM1 knockdown reveals that store-operated Ca2+ channels located close to

Endoplasmic reticulum-mitochondrial-associated membranes (ER-MAM, or ER-MAM) are ER-contiguous membranes associated with mitochondria (Rusinol et al., 1994) A unique mitochondria-associated membrane fraction from rat liver has a high capacity for lipid synthesis and contains pre Golgi secretory proteins including nascent lipoproteins. *J. Biol. Chem.* 269:27494-27502) that constitute a physical bridge that connects the ER to mitochondria (Csordas et al., 2006) Structural and functional features and significance of the physical linkage between ER and mitochondria. *J. Cell Biol.* 174:915-921; Rusinol et al., (1994) A unique mitochondria-associated membrane fraction from rat liver has a high capacity for lipid synthesis and contains pre Golgi secretory proteins including nascent lipoproteins. *J. Biol. Chem.* 269:27494-27502).


[00430] A few non enzymatic proteins are also concentrated in ER-MAM, including the IP3 receptor (IP3R) (Hajnoczky et al., 2002) Old players in a new role: mitochondria-associated membranes, VDAC, and ryanodine receptors as contributors to calcium signal propagation from endoplasmic reticulum to the mitochondria. *Cell Calcium* 32:363-377), autocrine motility factor receptor (AMFR; an E3 ubiquitin ligase that targets ER proteins for proteasomal degradation (Registre et al, 2004) The gene product of the gp78/AMFR ubiquitin E3 ligase cDNA is selectively recognized by the 3F3A antibody within a subdomain of the endoplasmic reticulum. *Biochem. Biophys. Res. Commun.* 320:1316-1322), and apolipoprotein E (ApoE) (Csordas et al, 2006) Structural and


[00432] PSI and PS2. The results and analysis described herein that relate to the effect of PSI on ER-MAM integrity (for example, results relating to PSI mutations, overexpression of PSI and reduced expression) also apply to PS2. For example, the effects on ER-MAM integrity that occur as a result of loss or reduction of PSI function, also occur where PS2 function is lost or reduced.

[00433] Morphology of AD Fibroblasts. Skin fibroblasts from patients with FAD due to a mutation in PSI are significantly smaller, more "spherical" and less elongated, and have an altered perinuclear distribution of mitochondria as compared to control fibroblasts. Notably, these three properties are consistent with the mislocalization of mitochondrial as described herein. PSI -mutant fibroblasts are smaller than age- and sex-matched control fibroblasts (Fig. 1). This was confirmed in a more objective way by trypsinizing PSI and
control fibroblasts to de-attach them from the plates, and then analyzing them by fluorescent-activated cell sorting (FACS). This analysis confirmed that PSI fibroblasts are significantly smaller than controls, and that unattached PSI fibroblasts are significantly less elongated than controls (i.e. they have a smaller aspect ratio) (Fig. 2). This sphericity may occur if organelles are no longer attached to microtubules.

[00434] Presenilins are ER-MAM-associated protein. Plasma membrane (PM), ER, and crude mitochondria (CM) was isolated from mouse liver, and then fractionated CM into a ER-MAM fraction and a purified mitochondrial fraction (Vance et al., 1997) Phosphatidylethanolamme N-methyltransferase from liver. Biochim. Biophys. Acta 1348:142-150; the purity of the fractions was confirmed by Western blotting. Western blot analysis was performed on ER, ER-MAM, and mitochondrion isolated from mouse liver and brain using relevant antibodies for the 3 compartments (SSR-a, ACAT1, and NDUFA9, respectively) as well as antibodies that recognize both the N- and C-terminal fragments of PSI (Fig. 3). The majority of PSI (both NTF and CTF) in both tissues was present in the ER-MAM fraction, similar to the pattern seen for ACAT1, a known ER-MAM-associated protein. The localization of PSI to that of PEMT, another known ER-MAM-associated protein, was compared using immunocytochemistry and staining with MitoTracker Red (MTred) to visualize mitochondria. Using cold methanol (MeOH) to dehydrate and fix the cells, it was found that PEMT colocalized with MTred staining (Fig. 4D). This result is consistent with the fact that PEMT is enriched in a compartment bridging mitochondria and ER. The colocalization of PEMT with MTred was most pronounced in the region around the nucleus (red arrowheads in Fig. 4D). This results shows that that ER-MAM is located predominantly in the perinuclear region under these fixation conditions. PSI also co-localized with MTred, also predominantly in the perinuclear region (Fig. 4C). Double-staining of cells for both PSI and PEMT showed that the two proteins colocalized almost exactly (Fig. 4E). These results show that PSI, like PEMT and ACAT1, is a ER-MAM-associated protein.

[00435] MAM and mitochondria in fibroblasts FADPS1 or FADPS2 patients. Because presenilin is located in a domain connecting ER with mitochondria, subcellular fractionation of control and FADPS1 (A246E mutation) fibroblasts was performed and total protein recovered in ER, ER-MAM, and mitochondrial fractions was measured to determine if pathogenic mutations in PSI affect these compartments, qualitatively or quantitatively. A significant decrease in the amount of ER-MAM protein, increase in ER-MAM function and a
significant increase in the amount of mitochondria in FAD<sup>PSI</sup> cells vs. controls was observed (Fig. 5).

[00436] The morphology and distribution of MTred-labeled mitochondria in control and FAD<sup>PSI</sup> fibroblasts (mutations A246E and M146L) was examined. To define cell boundaries, the microtubule cytoskeleton was visualized by indirect immunofluorescence with anti-tubulin antibodies in the same cells. Mitochondria in PSI-mutant fibroblast lines were more concentrated around the nucleus than were mitochondria in controls and fewer mitochondria were observed at the extremities of FAD<sup>PSI</sup> cells (representative result in Fig. 6A). This effect was quantitated by measuring the intensity of the MTred signal in the periphery of the cell. A circle of uniform size that occupied about 2/3 of the cell's area and also encompassed the nucleus was drawn and the amount of MTred signal outside the circle was measured. Using this approach, it was confirmed that there indeed were significantly fewer mitochondria in the cells' extremities in FAD<sup>PSI</sup> cells as compared to controls (Fig. 6C). The proportion of MAM in the cells was increased significantly (Fig. 6). These findings support the concept that PSI contributes to the stabilization of MAM. This "perinuclear" result is consistent with a defect in microtubular transport of mitochondria to the edges of the cells.

[00437] Besides an altered subcellular distribution, the mitochondria in FAD<sup>PSI</sup> cells also had an altered morphology. Whereas mitochondria in control fibroblasts had an elongated, tubular morphology, mitochondria in patient fibroblasts were more punctate (Fig. 6B). The FAD<sup>PSI</sup> cells showed no obvious deficit in respiratory chain function. To reproduce the mitochondrial distribution defect observed in FAD<sup>PSI</sup> patient cells, COS-7 cells were transfected with a construct expressing wild-type PSI or the A246E mutation (. 7). Visualization of mitochondria and the microtubule cytoskeleton in transfected cells showed that mitochondria in the cells over-expressing mutant PSI, but not control cells, accumulated in the perinuclear region of the cell. This is a phenotype similar to that observed in FAD<sup>PSI</sup> cells.

[00438] Small hairpin RNA (sh-RNA) technology was used to knock down PS1 expression in mouse embryonic fibroblasts to reproduce the mitochondrial mislocalization phenotype. The "perinuclear" phenotype observed in cells that overexpress mutant PSI or in cells from FAD<sup>PSI</sup> patients was recapitulated using cells in which PSI expression was reduced by about 75% (Fig. 8E-F). The specificity of the shRNA primer was confirmed by
transducing a mismatch shRNA, which did not alter the mitochondrial distribution or morphology.

[00439] These results show that a pathogenic problem in FAD due to mutations in presenilin is the mislocalization of mitochondria in affected cells. Presenilins are part of the machinery required for the reversible, kinesin-mediated, binding of mitochondria to microtubules, and that mutations in presenilins cause mitochondria to dissociate from microtubules. Mitochondria are key players in FAD, and dysfunction of mitochondria in FAD may include mislocalization and ATP synthesis defects, increased ROS, and elevated Ca$^{2+}$ homeostasis. Since mitochondria are the main source of energy in the cell, failure to bind to microtubules efficiently can result in energy deficits in those parts of the cell that are relatively devoid of organelles. This mislocalization of mitochondria need not be deleterious in most cells, which are essentially "spherical" (i.e. they are not diffusion-limited for ATP), but can be more problematic in elongated neurons, which require that mitochondria travel vast distances along microtubules in order to provide ATP for energy-intensive processes at, for example, synaptic junctions.

[00440] **ApoE and APP Are Present in ER-MAM.** The enrichment of ApoE in ER-MAM was noted more than 15 years ago (Rusinol et al., (1994) A unique mitochondria-associated membrane fraction from rat liver has a high capacity for lipid synthesis and contains pre Golgi secretory proteins including nascent lipoproteins. *J. Biol. Chem.* 269:27494-27502; Vance JE (1990) Phospholipid synthesis in a membrane fraction associated with mitochondria. *J. Biol. Chem.* 265:7248-7256). In addition to confirming that ApoE is enriched in ER-MAM (Fig. 9) the results described herein show that APP is also present in abundant amounts in ER-MAM (Fig. 9). These findings show that ER-MAM is involved not only in familial AD, but in sporadic AD as well.

[00441] The findings that presenilins are enriched in ER-MAM, that presenilins affect the stability of this compartment, and that presenilins affect the distribution of mitochondria which can be associated with ER-MAM, point to an additional role for presenilins in the development of AD. Numerous hypotheses have been put forward to explain the etiology and progression of the disease. Foremost among these are hypotheses invoking β-amyloid and tau, but alterations in cholesterol, glucose, and lipid metabolism, and in calcium homeostasis, play roles in AD pathogenesis. ER-MAM harbors proteins involved in lipid metabolism (e.g. fatty acid-CoA ligase (Lewin et al, (2002) Rat liver acyl-CoA
Given these functions, alterations in ER-MAM structure, function, and integrity can explain many of the biochemical changes found in cells and tissues from AD patients. Moreover, because PS1, ApoE and APP are present in ER-MAM, the familial and sporadic forms of AD can be related in a fundamental way, in which altered ER-MAM integrity is the common denominator. The results described herein take AD research in a new direction, as it predicts a cause-and-effect relationship between altered ER-MAM integrity, mitochondrial dynamics, and neurodegeneration. This relationship is not unreasonable, since mitochondrial mislocalization plays a role in the pathogenesis of other neurodegenerative diseases. These include (1) hereditary spastic paraplegia type 7, due to mutations in paraplegin (SPG7), a mitochondrial AAA protease, which is associated with abnormal mitochondria and impaired axonal transport (Ferreirinha et al., 2004) Axonal degeneration in paraplegin-deficient mice is associated with abnormal mitochondria and impairment of axonal transport. J. Clin. Invest. 113:231-242); (2) Charcot-Marie-Tooth disease type 2A, a peripheral neuropathy caused by mutations in the kinesin motor KIF1B and in mitofusin 2 (MFN2; a mitochondrial outer membrane protein required for organellar fusion); both cause altered axonal transport (Baloh et al., 2007) Altered axonal mitochondrial transport in the pathogenesis of Charcot-Marie-Tooth disease from mitofusin 2 mutations. J. Neurosci. 27:422-430; Zhao et al., 2001) Charcot-Marie-Tooth disease type 2A caused by mutation in a microtubule motor KIF1Bp. Cell 105:587-597); (3) Charcot-Marie-Tooth disease type 4A, due to mutations in ganglioside-induced differentiation associated protein 1 (GDAP1), a mitochondrial outer membrane protein that regulates organellar morphology (Niemann et al., 2005) Ganglioside-induced differentiation associated protein 1 is a regulator of the mitochondrial network: new implications for Charcot-Marie-Tooth disease. J. Cell Biol. 170:1067-1078); and (4) autosomal-dominant optic atrophy, due to mutations in OPA1 (a mitochondrial dynamin-related protein that interacts with mitofusin-1 (Cipolat et al., 2004) OPA1 requires mitofusin 1 to promote mitochondrial fusion. Proc. Natl. Acad. Sci. USA 101:15927-15932)) and which is characterized by a maldistribution of mitochondria in affected cells (Delettre et al., 2000) Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy. Nat. Genet. 26:207-210). The results described herein are supported by (1) the observation that a PS1 mutation (M146V) in a mouse PS1 knock-in model impairs axonal transport and also increases tau phosphorylation (Pigino et al., 2003) Alzheimer’s presenilin 1 mutations impair kinesin-based axonal transport. J. Neurosci. 23:4499-4508), (2) the finding of axonal defects, consisting of the

[00443] Example 3: Mitochondrial Maldistribution

[00444] The result that mitochondria are mislocalized in AD indicates a cause-and-effect relationship between mitochondrial mislocalization and neurodegeneration, as opposed to a model in which APP and amyloid are primary determinants in the pathogenesis of FAD due to presenilin mutations. The accumulation of β amyloid, tau, neurofibrillary tangles, and other sequellae of APP processing are downstream events.

[00445] The results described herein show that (1) PS1 is targeted to a specific compartment of the ER that is intimately associated with mitochondria, called ER-mitochondria-associated membranes (ER-MAM, or ER-MAM), (2) there is a significant change in the amount of ER-MAM protein in cells from FAD^{PS1} patients, and (3) there are defects in mitochondrial distribution and morphology in fibroblasts from FAD^{PS1} patients and in shRNA-mediated PS1-knockdown cells: mitochondria in these cells fail to reach the cell periphery and exhibit abnormal fragmentation. ER-MAM has known functions in lipid metabolism (including ApoE) and glucose metabolism, and in calcium homeostasis, all of which are functions known to be compromised in Alzheimer's disease (AD). These results show that mutations in presenilins inhibit mitochondrial distribution and neuronal transmission through effects on mitochondrial ER interactions. Because Ca^{2+} regulates the attachment of mitochondria to microtubules, the defects in mitochondrial distribution
observed FAD$_{PS1}$ cells can be due to defects in ER-MAM-mediated calcium homeostasis that alter axonal mitochondrial transport. Alternatively, because ER-MAM has been shown to contribute to the anchorage of mitochondria at sites of polarized cell surface growth, accumulation of mitochondria in the nerve terminal can be compromised in presenilin mutants. These two models are not mutually exclusive. This analysis has been performed to determine the mechanism underlying defects in mitochondrial distribution in presenilin mutants, and to address the role of ER-MAM-localized presenilin.

[00446] To determine if the mitochondrial maldistribution phenotype is clinically relevant cultured fibroblasts, mitochondrial distribution in neurons and in other cells and tissues from humans and from transgenic mice harboring pathogenic mutations in presenilin can be examined. Brain tissue from autopsies of FAD patients with presenilin mutations can be examined for mitochondrial distribution defects. A correlation between ApoE allele status, the mitochondrial distribution phenotype and the amount of communication between the ER and mitochondria in patient cells and tissues can be determined.

[00447] To determine the role of presenilin in ER-MAM blue-native gels, immunoprecipitation, and protein identification techniques can be used to determine if ER-MAM-localized presenilin interacts with other partners in the ER-MAM subcompartment and to determine the effects of mutations in presenilin binding partners on ER-MAM localization.

[00448] To determine how mutant presenilin causes mitochondrial maldistribution the effect of presenilin mutations on anterograde and retrograde axonal transport of mitochondria, on retention and accumulation of mitochondria in nerve terminals, and on the dynamics of mitochondrial fusion and fission can be examined using mitochondrially-targeted photo-activatable fluorescent probes ("mitoDendra") and live-cell imaging of neuronal cells. In order to determine the relevance of these observations to AD, these studies can be conducted in primary neuronal cells derived from normal, FAD$_{PS1}$ and FAD$_{PS2}$ mice of different ages.

[00449] Example 4: Role of Presenilin in Mitochondrial Mislocalization

[00450] Mutated presenilins can be transfected into normal fibroblasts in order to recapitulate the morphological abnormalities observed in FAD$_{PS1}$ or FAD$_{PS2}$ fibroblasts (obtained from the Coriell Cell Repository). Since FAD is a dominant disorder, both the wild-type and mutant presenilin alleles are present in these cells. The normal and mutated presenilin (for example, E280A mutation in PS1) alleles from this cell line can be amplified
and subcloned it into a mammalian expression vector, such as pCDNA3.1 (Stratagene). In order to be sure that the presenilin expressed from the transfected constructs is targeted to mitochondria, a His6 epitope tag can be attached to the C-terminus of the polypeptide, and anti-His-tag immunohistochemistry can be used to confirm the subcellular localization to mitochondria and to adherens junctions. Western blots and in-vitro importation assays can be performed to determine sub mitochondrial localization. Normal fibroblasts can be transiently co-transfected with a 10:1 ratio of the presenilin constructs and a construct encoding mitochondrialy-targeted GFP, so that the cells containing "green" mitochondria can also be expressing the presenilin construct to allow investigation of mitochondrial morphology (i.e. on the green mitochondria) without having to distinguish between the morphology of transfected vs. untransfected cells.

**Example 5: Mitochondrial mislocalization in FAD brain**

Brain tissue from autopsies of FAD patients with presenilin mutations can be examined to see if morphological abnormalities can be observed in neurons similar to those observed in fibroblasts.

**Example 6: Reversal of the mitochondrial mislocalization phenotype**


**Example 7: Characterization of the Mitochondrial Maldistribution Phenotype**
Further characterization of ER-MAM in neurons. The immunohistochemical and Western blot data show that presenilins are ER-MAM-associated proteins. The association of presenilin with ER-MAM and the disposition of this compartment in neurons can be further characterized using antibodies to the ER-MAM markers PEMT, PACS2, and FAC4L4 (Abgent AP2536b). ER-MAM has not been studied in neurons. Such analysis can contribute to the general understanding of neurons, and the effect of disrupting ER-MAM on neuronal function.

Example 8: Analysis of other Mutations

The studies described herein were carried out on fibroblasts isolated from FADPS1 patients with the A246E and M146L mutations. Fibroblasts from FAD patients with other PS1 mutations (lines EB [G209V], GF [I143T], WA [L418F]), and WL [H163R]), a fibroblast line carrying a PS2 mutation (line DD [N141I]) and a line carrying a pathogenic ("Swedish") mutation in APP can be studied as described herein.

Example 9: PS1 is enriched in the ER-MAM

Various cells were stained for mitochondria (using the mitochondrion-specific dye MitoTracker Red [MT Red; Molecular Probes]) and immunohistochemistry was performed to detect PS1 (Abeam abl0281). Initial investigations using "standard" immunohistochemistry (i.e. paraformaldehyde (PF) fixation followed by digitonin and/or Triton X-100 [TX-100] permeabilization of the cells prior to application of antibodies) revealed nonspecific staining of numerous membranous compartments (e.g. ER, Golgi, plasma and nuclear membranes), similar to the results reported by others; a representative result for monkey COS-7 cells is shown in Fig. 4A. When the permeabilization technique was modified by omitting the treatment with TX-100 and by fixing the cells with either cold methanol (MeOH) alone (Fig. 4B) or with PF followed by MeOH, a different result was obtained where PS1 co-localized with the MT Red stain, predominantly in the perinuclear region. PS1 was also present diffusely in areas that were devoid of mitochondria (fainter green regions in Fig. 4B, asterisks); presumably these are plasma membrane, ER, and/or Golgi.

To confirm that PS1 is a ER-MAM-enriched protein, immunocytochemical localization of PS1 in human fibroblasts was compared with that of PEMT, an authentic ER-MAM protein (Cui et al. (1993) J. Biol. Chem. 268:16655-16663; Rusinol et al. (1994) J. Biol. Chem. 269:27494-27502). PEMT co-localized with a subset of
mitochondria, as visualized by staining with MT Red, as expected for a protein that is localized in a compartment that serves as a bridge between mitochondria and ER (i.e. ER-MAM) (Fig. 4D). The colocalization with MT Red was most pronounced in the region around the nucleus, indicating that the ER-MAM subcompartment is located predominantly in the perinuclear region of the cell. Like PEMT, PSI also colocalized with MT Red, and also predominantly in the perinuclear region (Fig. 4C). Finally, double staining of cells for both PSI and PEMT shows that they co-localized almost exactly (Fig. 4E).

[00462] PSI staining was also observed at adherens junctions in the plasma membrane in confluent COS-7 (Fig. 14A) and in human 293T and mouse 3T3 cells, also as seen by others (Georgakopoulos et al. (1999) Mol. Cell. 4:893-902; Marambaud et al. (2002) EMBO J. 21:1948-1956), confirming a known location for PSI even when cells were fixed in MeOH. Since PSI is associated with neurodegeneration, PSI localization was studied in primary rat neurons. PSI co-localized more with MT Red signal that is perinuclear and within the cell body compared to processes away from the cell body (Fig. 14B).

[00463] The use of TX-100 to permeabilize the cells prior to immunohistochemical detection has a profound effect on PSI localization. This finding is consistent with the observation that TX-100 permeabilization alters immunolocalization of mitochondrial proteins (Melan MA, Sluder G (1992) J. Cell Sci. 101:731-743; Brock et al. (1999) Cytometry 35:353-362). Equally important, the results described herein indicate that PSI localizes to a subset of perinuclear mitochondria in neurons and non-neuronal cells. Since PSI is not targeted to all mitochondria and since import of PSI into mitochondria in an in-vitro import assay was not detected, and since it has a subcellular distribution essentially identical to that of PEMT, the results described herein show that PSI is not a mitochondrial-targeted polypeptide, but is rather an ER-MAM polypeptide that is "mitochondria-associated" under some circumstances. The immunocytochemical data support a localization of PSI to ER-MAM. However, since there is no apriori reason to believe that MeOH fixation without TX-100 gives a more accurate result than methods using TX-100, subcellular fractionation was used to evaluate the association of PSI with ER-MAM. Plasma membrane (PM), crude mitochondria (CM), and ER was isolated as described (Stone SJ, Vance JE (2000) J. Biol. Chem. 275:34534-34540; Vance JE (1990) J. Biol. Chem. 265:7248-7256), and crude mitochondria was further fractionated by isopycnic centrifugation (Vance et al. (1997) Biochim. Biophys. Acta 1348:142-150) into a ER-MAM and a purified mitochondrial fraction. The fractions were evaluated by Western blot analysis using antibodies to cadherin
(CDH2; marker for PM), calnexin (CANX; for ER), signal sequence receptor aSSRI; for ER), Golgi matrix protein GM130 (GOLGA2; for Golgi), ACAT1, G6PC, and PEMT (for ER-MAM [and to a lesser extent, ER]), and the NDUFA9 subunit of complex I of the respiratory chain (for mitochondria) (Fig. 15).

The analysis indicated that the ER-MAM fraction is distinct from ER or purified mitochondrial fractions. Specifically, the ER-MAM fraction was enriched for PEMT, G6PC, and ACAT1, known ER-MAM markers. Conversely, marker proteins for the PM, Golgi, ER and mitochondria were selectively depleted from the ER-MAM fraction (Fig. 10).

Analysis by Western blot of the ER, ER-MAM, and mitochondria fractions from mouse liver and brain showed that the majority of PS1 was present in the ER-MAM fraction, similar to the pattern seen for ACAT1 (Figs. 15A-15B). This finding, together with immunohistochemistry studies, indicate that PS1 is localized to a subcompartment of mitochondria associated with ER, i.e., ER-MAM.

Using a FRET-based assay (R&D Systems #FP003) on subcellular fractions from mouse, the γ-secretase specific activity was observed to be about 5 times higher in the ER-MAM than in the ER, in both liver and brain (Fig. 19). This result shows that that PS1 is enriched in ER-MAM.

Example 10: Presenilins are enriched in mitochondria-associated membranes

Plasma membrane (PM), crude mitochondria, and ER were isolated from mouse brain, and fractionated crude mitochondria further by isopycnic centrifugation (Vance et al, Biochim. Biophys. Acta 1997, 1348:142-150) into a MAM fraction and a purified mitochondrial fraction. Each of these fractions were evaluated by Western blot analysis, using antibodies to Na,K-ATPase as a marker for PM, to SSR as a marker for ER, to Golgi matrix protein GM130 (GOLGA2) as a marker for Golgi, to IP3R3 as a marker for MAM, and to the a subunit of ATP synthase (ATP synthase-a) as a marker for mitochondria (Figure 28A). All five markers were enriched in their respective compartments, but low levels of mitochondrial ATP synthase-a were also present in the plasma membrane. ATP synthase-a has been found in this compartment by others (Bae et al, Proteomics 2004, 4:3536-3548). The MAM fraction was enriched for IP3R3, a known MAM marker, (Mendes et al, . Biol. Chem. 2005, 280:40892-40900) confirming separation of MAM from bulk ER and mitochondria to a degree sufficient for further analysis. The amount of protein recovered in each of the
subcellular fractions analyzed from whole mouse brain was quantitated. Of the total amount of protein recovered in the ER fraction, ~13%±0.3% (n=6) was in the MAM subfraction. This value reflects the analysis of total mouse brain, and can vary in different brain regions and in different tissues.

[00469] Western blot analysis was then performed on these same fractions from mouse brain, using antibodies against PS1 and PS2 (Figure 28B). PS1 was found in the plasma membrane/Golgi fractions, as reported previously, (De Strooper et al, J. Biol. Chem. 1997, 272:3590-3598) however, as described herein, PS1 is essentially an ER-resident protein (Figure 28B). However, within the ER, PS1 was not distributed homogeneously, but rather was enriched in ER membranes that are in close contact with mitochondria (i.e. MAM) (Figure 28B). Like PS1, PS2 was also enriched in the MAM (Figure 28B). Analysis of the blots revealed that the amount of PS1 was enriched by 5- to 10-fold in MAM over that in "bulk" ER (n=12).

[00470] The various subcellular fractions of mouse brain where then assayed for the presence and amount of γ-secretase activity, using two different assays (Figures 29A and 29B). Most of the γ-secretase activity was detected in MAM compared to the other fractions assayed, showing not only that PS1 and PS2 are enriched in this fraction, but that the other components of the γ-secretase complex - APH1, NCT, and PEN2 - are present there as well (Sato et al, J. Biol. Chem. 2007, 282:33985-33993). Using Western blotting, it was observed that those three polypeptides were enriched in the bulk ER, but were present in significant amounts in the MAM as well. Why the amount of the various γ-secretase components are not distributed proportionally in the two compartments can be a reflection of the different steps in the assembly pathway for the holoprotein (Spasic et al, J. Cell Sci. 2008, 121:413-420). Moreover, APP itself was also present in high amounts in the MAM (Figure 2B). Thus, MAM contains both the enzymatic activity to cleave APP (i.e. γ-secretase) and the APP substrate itself. The localization of γ-secretase activity in MAM could help explain the unexpected presence of Aβ in mitochondria (Du et al, Nat. Med. 2008, 14:1097-1105)

[00471] To further confirm that PS1 is a MAM-enriched protein, the immunocytochemical localization of PS1 in human fibroblasts was compared with that of FACL4, a known MAM-localized protein (Lewin et al, Arch. Biochem. Biophys. 2002, 404:263-270). Cells were stained with MT Red and then detected FACL4 by immunocytochemistry (Figure 30A). FACL4 immunostain (green) was found to be "co-
localized" with MT Red (red), but only partially: the "co-localization" was most predominant in the region around the nucleus (yellow arrowhead in Figure 30A), but not in the more distal regions of the cell (red arrowhead in Figure 30A). This result shows that the much of the yellow signal reflected the juxtaposition of MAM with mitochondria (see enlarged merge panel at right in Figure 30A). Like FACL4, PSI partially co-localized with MT Red, and also predominantly in the perinuclear region (Figure 30B). The co-localization of PSI with MT Red in the perinuclear region was revealed to actually consist of small discrete regions of PSI immunostain apposed to discrete MT Red-positive regions (enlarged merge panel at right in Figure 30B), a pattern highly similar to that observed with FACL4 (Figure 30A) and with the sigma-1 receptor, another MAM-resident protein (Hayashi et al, Cell 2007, 131 :596-610). This result is also consistent with the finding that PSI was not imported into mitochondria in an in vitro import assay. Finally, when cells were double-stained for both PSI and FACL4, the two proteins co-localized almost exactly, even at enlarged magnification (Figure 30C). These results show that both PSI and FACL4 reside in the same compartment, namely MAM. Quantitative analysis of the degree of co-localization confirmed these conclusions. In particular, the co-localization of PSI with MT Red (as a decimal fraction) was 0.51±0.08, which was not statistically different than the value of 0.47±0.05 for the co-localization of FACL4, an authentic MAM protein, with MT Red. The quantitative data support the immunocytochemical results, namely, that PSI is not a mitochondrial protein, but resides in a compartment adjacent to mitochondria, in a manner essentially identical to that of FACL4 (i.e. MAM).

[00472] The immunocytochemical results were confirmed in other cell types, including primary rat cortical neurons and mouse 3T3 cells. Importantly, a similar result was obtained using immunocytochemistry to detect human PS2 in mouse cells (Figure 30D). Finally, besides the immunocytochemical localization to MAM, PSI staining at adherens junctions in the plasma membrane was also observed in confluent COS-7 (Figure 30E) and in human 293T and mouse 3T3 cells.

[00473] Taken together, the Western blotting, γ-secretase activity, and immunocytochemistry results show that PSI and PS2 are indeed MAM-enriched proteins, in both neuronal and non-neuronal cells. The difference between the results described herein and reports in which presenilins were found in fractions enriched in markers characteristic of other subcellular compartments, such as ER (Annaert et al, J. Cell Biol. 1999, 147:277-294), Golgi (Annaert et al, J. Cell Biol. 1999, 147:277-294), the trans-Golgi network (Siman et al,

[00474] Example 11: Mitochondrial dynamics in cells expressing mutated PSI

[00475] To determine if PSI has functionally significant interactions with ER-MAM, the morphology and distribution of MT Red-labeled mitochondria in fibroblasts from a control and an FAD\textsuperscript{PSI} patient (mutation A246E [Coriell AG06840]) was studied. To define cell boundaries, the microtubule cytoskeleton (with anti-tubulin) in the same cells was also visualized.

[00476] Overexpression of mutant PSI in stably-transfected transfected COS-7 cells showed that mitochondria in the cells over-expressing mutant PSI, but not control cells, accumulated in the perinuclear region of the cells (Fig. 7), similar to the results observed in FAD\textsuperscript{PSI} patient and PSI-KD cells (as described herein).

[00477] Mitochondria in PSI-mutant fibroblasts were more concentrated around the nucleus than were mitochondria in controls, with fewer mitochondria at the extremities of FAD cells (Fig. 8A), and had an altered, more punctate, morphology (Fig. 8C). This effect was quantitated by measuring the intensity of the MT Red signal in the extremities of the cells to confirm that there were significantly fewer mitochondria in the cells’ extremities in FAD cells vs. controls (Fig. 8B). ER-MAM protein in the cells was reduced significantly
when PS1 is mutated but the amount of communication was increased with mutated PS1 (Fig. 8D). This result shows that when PS1 is mutated, the ER-MAM connections are tighter relative to the wild-type PSI condition.(Fig. 8D). Given that protein is used a surrogate marker, the lower amount of protein in the mutated SP1 condition is correlated with an increase in the level of non-proteinaceous material in ER-MAM, thereby resulting in a reduction in the density of ER-MAM. Thus, these results show that PS1 contributes to the destabilization of ER-MAM.

[00478] Small hairpin RNA (sh-RNA) technology was used to reproduce the mitochondrial maldistribution phenotype by knocking down PS1 expression in mouse embryonic fibroblasts (MEFs). The perinuclear phenotype was recapitulated using cells in which PS1 expression was reduced by >75% (Fig. 8E,F).

[00479] FAD$^{PS1}$ is a dominant disorder, but the exact nature of the dominant effect is unclear. Reproduction of the mitochondrial distribution defect in cells in which PS1 had been knocked down by shRNA shows that the mitochondrial maldistribution phenotype can be due to haploinsufficiency rather than a gain-of-function effect of the PS1 mutation (see also Giannakopoulos et al. (1999) Acta Neuropathol (Berl) 98:488-492; Shen J, Kelleher RJ, III (2007) Proc. Natl. Acad. Sci. USA 104:403-409).

[00480] A finding that mutations in PS1 cause haploinsufficiency rather than a gain of function is highly relevant to treatment strategies for FAD$^{PS1}$. PS1 expression was knocked down by >75% in CCL131 mouse neuroblastoma cells (Fig. 20). The cells were transfected stably with control or PS1 knockdown constructs, differentiated with retinoic acid for 3 days, stained with MT Red and anti-tubulin, and were analyzed in the Imaging Core. In mismatched control (M3) cells, mitochondria were distributed relatively uniformly and densely along the processes (Fig. 20, brackets) and were enriched in varicosities, especially at branch points (Fig. 20, arrowheads). In siRNA-treated cells, however, there was a severely reduced number of mitochondria in cell processes, which was confirmed by scanning of the MT Red intensity along the length of the processes (Fig. 20, right panels) This result indicates that the alterations in mitochondrial dynamics observed in fibroblasts isolated from FAD$^{PS1}$ patients and in PS1-KD MEFs can operate in neuronal tissue as well. This finding can be confirmed in neurons isolated from PS1-mutated mice, as well as in PACS2-knockout mice.
While mutations in PS1 cause a mitochondrial mislocalization phenotype in fibroblasts and neuroblastoma cells, it was not clear if a similar or related phenotype is present in brain, the clinically relevant tissue in FAD. Autoptic brain tissue from patients was obtained and analyzed. The brain tissue was obtained from a sample of hippocampus from the autopsy of a patient with FAD$^{PS1}$ (A434C mutation) (Devi et al. (2000) Arch. Neurol. 57:1454-1457). Immunohistochemistry was performed to detect the FeS subunit of complex III of the mitochondrial respiratory chain in the CA1 region of the hippocampal formation (Fig. 21). There were two observations compared to control: (1) The mitochondria were concentrated in the perinuclear region of the neurons, often forming a "ring" of immunostain around the nucleus, and (2) there was an apparently corresponding absence of immunostain in the distal regions of the cell body.

These findings are in accord with data on cells in tissue culture and are consistent with the finding of axonal transport defects in PS1 transgenic mice (Pigino et al. (2003) J. Neurosci. 23:4499-4508). Analysis of more brain samples will be carried out using methods described herein and methods known to those skilled in the art. Taken together, these data indicate that mutations in PS1 have a profound effect on mitochondrial morphology and distribution in somatic and neuronal cells.

**Example 12: Biochemical function in PS1-mutant cells**

To establish feasibility, initial investigations focused on analysis of respiratory chain function in mitochondria isolated from Percoll-purified mouse brain mitochondria (MBM) from one PS1-transgenic (Tg) mouse and a wild type littermate (Duff et al. (1996) Nature 383:710-713). Isolated mitochondria were well-coupled (respiratory control index [RCI] >9) and exhibited normal rates of the phosphorylating (State 3) and resting (State 4) respiration, and high phosphorylation efficiency (ADP:0 >3) (Table 5).

Table 5. Respiration rates of MBM at 37°C. The phosphorylating (State 3) respiration was initiated by the addition of 200 nmol ADP to the mitochondrial suspension. RCI and the ADP:0 ratio were calculated by conventional procedures.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>State 3</th>
<th>State 4</th>
<th>RCI</th>
<th>ADP:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-Type</td>
<td>298</td>
<td>36</td>
<td>9.3</td>
<td>3.5</td>
</tr>
<tr>
<td>PS1 Tg</td>
<td>292</td>
<td>31</td>
<td>9.7</td>
<td>3.9</td>
</tr>
</tbody>
</table>
Note that values above 3 are due to the contribution of the substrate level phosphorylation, as 2-oxoglutarate was included in the respiratory substrate mixture. However, the RCI and ADP:0 ratio in mitochondria from the PS1 Tg animal was higher than in WT mitochondria. Analysis of the activity of the respiratory chain and tricarboxylic acid cycle enzymes (Table 6) did not reveal significant differences between PS1-Tg and WT mitochondria, except that the activity of Complex I was higher in the PS1 transgenic mitochondria. As the difference is beyond the normal range of variability for these measurements, which is -10-15%, this requires further investigation in more mice. In spite of the higher Complex I activity in PS1-Tg mitochondria, there was no difference in the respiration rates supported by the oxidation of NAD-linked substrates (Table 5). Activity of Complexes III-V remain to be assessed.

Table 6. Enzyme activities of MBM. PDHC, pyruvate dehydrogenase complex activity by following the reduction of NAD+ by pyruvate; MDH, malate dehydrogenase activity by following the oxaloacetate-induced NADH oxidation, CS, citrate synthase. All activities in nmol (NADH, NAD+, DCIP, or DTNB, respectively) per min per mg mitochondrial protein.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Complex I</th>
<th>Complex II</th>
<th>PDHC</th>
<th>MDH (x100)</th>
<th>CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-Type</td>
<td>1314</td>
<td>440</td>
<td>52</td>
<td>35</td>
<td>750</td>
</tr>
<tr>
<td>PS1 Tg</td>
<td>1751</td>
<td>451</td>
<td>54</td>
<td>33</td>
<td>740</td>
</tr>
</tbody>
</table>

Western blot analyses (Fig. 22) demonstrated similar contents of Complex III in the PS1-Tg and WT mitochondria. The levels of cytochrome c and MnSOD were also similar, indicating an equal level of structural integrity of the isolated WT and PS1-Tg mitochondria, as cytochrome c is a marker of the intermembrane space and MnSOD is a marker of the matrix space. The content of a matrix antioxidant enzyme, GSH reductase, appeared somewhat elevated in the PS1 mitochondria. The monoclonal antibody to GSH reductase cross-reacted with an unknown protein of ~33 kDa, with a much more intense signal in the PS1-Tg animal that appeared to be specific to PS1-Tg mitochondria (Fig. 22). This can indicate an enhanced detoxifying capacity of these mitochondria toward H$_2$O$_2$ and lipid radicals, and is consistent with the results described herein of elevated reactive oxygen species (ROS) in mouse PS1 KO and PS1/PS2-dKO blastocysts and MEFs stained with MitoSox (Molecular Probes) (Fig. 23).

Data were generated on a single pair of matched WT and Tg mice, as an initial pilot study. The same analyses will be carried out on a statistically relevant group of
animals. This Tg mouse expresses PSl from three alleles: two WT mouse PSl alleles and the mutant human PSl transgene. Given that FAD$^{PS1}$ may be due to a haploinsufficiency, the bioenergetic "profile" of this Tg line may represent the smallest effect due to mutations in PSl. Analysis of mitochondria isolated from brain and cells from PS1/PS2 dKO mice, which have no WT PSl alleles, and from PACS2-KO mice in which ER-MAM function is compromised, will be even more informative.

**00488** Oxygen consumption was measured polarographically in PS1-knockdown (PSI-KD) 3T3 cells and in PSI-KO and PS1/PS2- dKO MEFs. No difference in $O_2$ consumption was observed in the KD cells, but a statistically significant 40% increase was observed in the dKO cells (Fig. 24A). Using HPLC, a reduction of about 40% in ATP synthesis in PSI-KD and PSI-KO cells was observed, and about 60% reduction was observed in the dKO MEFs (Fig. 24B). The finding of reduced ATP synthesis but normal respiratory chain activity can be connected to the increase in ROS in these cells and the increase in complex I activity that was observed in the Tg mice.

**00489** Example 13: Calcium Homeostasis

**00490** The close association between ER and mitochondria at the ER-mitochondrial interface is important for calcium signal propagation from IP3 receptors (IP3R) to the mitochondria (Csordas et al. (2006) J. Cell Biol. 174:915-921; Rizzuto et al. (1998) Trends Cell Biol. 8:288-292). Because the results described herein show preferential localization of PSl at the ER mitochondrial interface, the effect of PSl and PSl depletion on mitochondrial calcium signaling was evaluated.

**00491** Previous studies have shown PS depletion to cause changes in ER Ca2+ storage and in IP3R function (Smith et al. (2005) Cell Calcium 38:427-437; Ito et al. (1994) Proc. Natl. Scad. Sci. USA 91:534-538; Tu et al. (2006) Cell 126:981-993). Cytoplasmic Ca2+ ([Ca2+]c) was monitored simultaneously with mitochondrial matrix Ca2+ ([Ca2+]m) in 3T3 cells transfected with a PSl -scrambled (control) or PSl -specific knockdown (PSI-KD) shRNA constructs (>75% reduction in PSl). The cells were transfected with a non-ratiometric mitochondrial matrix-targeted Ca2+-sensitive fluorescent protein (inverse pericam (Zhang et al. (2008) BMC Neurosci. in press)) to record [Ca2+]m and were loaded with fura2/AM for ratiometric imaging of [Ca2+]c at 340/380 nm to record [Ca2+]c in single cells (Fig. 25). The cells were stimulated sequentially with ATP (to induce IP3R-mediated Ca2+ mobilization), with thapsigargin (Tg; an inhibitor of the SERCA to complete depletion
of Ca2+ from the ER into the cytosol), and finally with extracellular CaCl2 (to allow for store-depletion-induced Ca2+ entry into the cytosol). Addition of ATP evoked a cytosolic [Ca2+]c spike in both control and PS1-KD cells, but the [Ca2+]c spike was relatively large in the PS1-KD cells (n=7 experiments), a result consistent with a recent report on the effect of mutant PSI and PS2 on Ca2+ mobilization (Tu et al. (2006) Cell 126:981-993). Release of the residual ER Ca2+ by Tg and the store-depletion operated Ca2+ influx caused similar elevations in [Ca2+]c in both WT and PSI KD cells (Fig. 25A). Thus, the ER Ca2+ storage was greater, and allowed for larger IP3 induced Ca2+ mobilization in the PS1-KD cells. Simultaneous measurements of mitochondrial [Ca2+]m (Fig. 25B) showed a rapid transfer of the IP3-induced [Ca2+]c signal to the mitochondria. However, the [Ca2+]m signal was >2-fold higher in the PS1-KD cells (n=7). As expected, Tg and CaCl2 induced similar [Ca2+]m increases in both WT and PS1 KD cells (Fig 25B). Thus, IP3-dependent Ca2+ transfer to mitochondria was massively increased in the PS1-KD cells.

[00492] For measurements of mitochondrial matrix [Ca2+] ([Ca2+]m), the cells were transfected with a mitochondrial matrix targeted inverse pericam construct (Nagai et al.(2001) Proc. Natl. Acad. Sci. USA 98:3197-3202) by electro oration 24-48 h prior to the imaging experiment. Cells were preincubated in an extracellular medium as described (Yi et al. (2004) J. Cell Biol. 167:661-672; Duff et al. (1996) Nature 383:710-713). To monitor [Ca2+]c cells were loaded with 5µM Fura2/AM for 20-30 min in the presence of 200 µM sulfinpyrazone and 0.003% (w/v) pluronic acid at room temperature. Before start of the measurement the buffer was replaced by a Ca2+-free 0.25%BSA/ECM ([Ca2+] < 1µM). Coverslips were mounted on the thermo stated stage (35°C) of a Leica IRE2 inverted microscope fitted with a 40X (Olympus UApo, NA 1.35) oil immersion objective. Fluorescence images were collected using a cooled CCD camera (PXL, Photometries).

[00493] Excitation was rapidly switched among 340 and 380 nm for fura2 and 495 nm for pericam, using a 510 nm longpass dichroic mirror and a 520 nm longpass emission filter. For evaluation of [Ca2+]c, Fura2 fluorescence was calculated for the total area of individual cells. [Ca2+]c was calibrated in terms of nM using in vitro dye calibration. For evaluation of [Ca2+]m, the pericam-mt signal was masked. Recordings obtained from all transfected cells on the field (8-15 cells) were averaged for comparison in each experiment. Significance of differences from the relevant controls was calculated by Student's t test. Cells will be challenged with compounds that affect intracellular Ca2+ concentration, such as 300 nM bradykinin (which stimulates IP3-mediated Ca2+ release) and 5 µM ionomycin (a
Ca2+ ionophore that induces formation of Ca2+-permeable pores, leading to emptying of ER Ca2+ stores independent of IP3-mediated receptor activation (Nelson et al. (2007) J. Clin. Invest. 117:1230-1239)).

[00494] In summary, silencing of PSI caused an increase in the IP3-dependent Ca2+ mobilization and massive potentiation of the ensuing mitochondrial Ca2+ accumulation, confirming that PSI is an important regulator of Ca2+ storage in the ER. This result indicates that PSI exerts a major effect on ER-mitochondrial Ca2+ transfer, sensitizing mitochondria to permeabilization in FADPSI cells, leading to cell injury.

[00495] Example 14: Functional assays of MAM

[00496] One of the described functions of MAM is to regulate the transport of selected lipids from the ER into the mitochondria. For example, phosphatidylserine (PtdSer) moves from the MAM to mitochondria, where it is decarboxylated to phosphatidylethanolamine (PtdEtn); PtdEtn then moves back to the MAM, where it is methylated to phosphatidylcholine (PtdCho) (Fig. 31). Thus, the kinetics of trafficking of PtdSer from the MAM to mitochondria is a recognized measurement of MAM function (Schumacher et al. (2002) J. Biol. Chem. 277:51033). In one embodiment, a MAM function assay is based on the measurement of the incorporation of 3H-Ser into phospholipids, as described by Voelker (Schumacher et al. (2002) J. Biol. Chem. 277:51033). As shown in the schematic in Fig. 31, exogenously added serine (Ser) is incorporated into PtdSer in the MAM, via an exchange reaction in which serine replaces ethanolamine (Etn) in PtdEtn or choline (Cho) in PtdCho via the action of phosphatidylserine synthase 1 and 2 (PTDSS1 and PTDSS2 in humans), respectively. The resulting PtdSer is then transported from the MAM to mitochondria, where it is decarboxylated to PtdEtn by mitochondrial phosphatidylserine decarboxylase (PISD). The resulting PtdEtn is transported back to the MAM, where it can be methylated to PtdCho by phosphatidylethanolamine methyltransferase (PEMT). In the MAM activity assay, 3H-Ser is added to cells in medium lacking Etn but containing Cho, so that PtdSer is made from PtdCho via PTDSS1, but not from PtdEtn via PTDSS2, at least not initially, because there is no exogenous source of Etn to form PtdEtn via the Kennedy pathway. Thus, the only way PtdEtn can be made is via the MAM pathway, and the amount of 3H incorporated into 3H-PtdSer and 3H-PtdEtn is a measurement of MAM function.

[00497] Applying this technique to PSI mutant fibroblasts and to PSI knock-out (PS1-KO) mouse embryonic fibroblasts (MEFs) vs. controls, a significant increase in PtdEtn
synthesis was detected in PS1-mutant cells (Fig. 32D), reflecting an upregulated transport of PtdSer into mitochondria, and implying that defects in PS1 indeed affect MAM function. As a control, MEFs in which MAM-mitochondrial communication had been abrogated by knocking out PACS2 (Simmen et al. (2005) EMBO J 24:717) were shown to retain their ability to synthesize 3H-PtdSer but had decreased formation of PtdEtn (and PtdCho).

Example 15: Elevated cholesterol in patients with AD

Mutations in PS1 causing altered MAM function will also show altered cholesterol content. Moreover, if MAM function is reduced in PS1-mutant cells and tissues, cholesterol content can be increased concomitantly. MAM indeed contains high levels of cholesterol, both as free cholesterol and as cholesterol esters (Fig. 33A). Moreover, when the crude mitochondrial fraction from the brains of WT and PS1-knock-in mice (M146L mutation; courtesy of Mark Mattson; Guo et al. (1999) Nature Med 5:101) are examined, the amount of both total and free cholesterol was increased in the KJ vs. the WT mice (Fig. 33).

This result can be explained by the role of a key MAM protein, acyl-coA:cholesterol acyltransferase (ACAT1 [gene SOAT1]), which not only synthesizes cholesterol esters in the MAM, but also is important for the generation of Aβ by modulating the equilibrium between free and esterified cholesterol (Puglielli et al. (2005) Nature Cell Biol. 3:905; Puglielli et al. (2004) J. Mol. Neurosci. 24:93). In addition, that steroid biosynthesis can require ER-mitochondrial communication, across the MAM, as cholesterol must be imported from the ER into mitochondria, where it is converted into pregnenolone, which is then exported back to the ER for further steroid synthesis (e.g. testosterone and estradiol). Thus, tighter communication between the MAM and mitochondria could increase cholesterol biosynthesis and hence, Aβ production.

Example 16: Mitochondrial dynamics in PS1-mutant neuronal-like cells

Since AD is a brain disorder, PS1 expression was knocked down by >75% in CCL131 mouse neuroblastoma cells and stained the cells with MitoTracker Red and anti-tubulin (Fig. 34). In control cells, mitochondria were distributed relatively uniformly and densely along the processes (Fig. 34, brackets) and were enriched in varicosities, especially at branch points (Fig. 34, arrowheads). In PS1-knockdown (KD) shRNA-treated cells, however, there was a severely reduced number of mitochondria in cell processes, which was confirmed by scanning of the MT Red intensity along the length of the processes. This result shows that...
The alterations in mitochondrial dynamics observed in fibroblasts isolated from FAD<sup>PS1</sup> patients and in PS1-KD MEFs operate in neuronal tissue as well.

**Example 17: Mitochondrial maldistribution in AD brain**

A similar or related mitochondrial maldistribution phenotype is present in brain, the clinically relevant tissue in FAD. A sample of hippocampus was obtained from the autopsy of a patient with FAD<sup>PS1</sup> (A434C mutation). Immunohistochemistry was performed to detect the FeS subunit of complex III of the mitochondrial respiratory chain in the CA1 region of the hippocampal formation (Fig. 35). This analysis resulted in at least two observations (1) The mitochondria were concentrated in the perinuclear region of the neurons, often forming a "ring" of immunostain around the nucleus, and (2) there was a corresponding absence of immunostain in the distal regions of the cell body. Both results are consistent with a perinuclear localization of mitochondria in FAD<sup>PS1</sup> brain. These findings are similar to results on cells in tissue culture and are consistent with the finding of axonal transport defects in PS1 transgenic mice (Stokin et al. 2005 Science 307:1282) and in human SAD patients (Stokin et al. 2005 Science 307:1282; Wang X, et al. 2008 Am. J. Pathol. 173:470).

**Example 18: ApoE and APP are also present in MAM**

ApoE activity is enriched in MAM (Vance 1990 J. Biol. Chem. 265:7248). ApoE protein is enriched in MAM (~3-fold over that in ER) (Fig. 36). In addition, APP is also present in abundant amounts in MAM (Fig. 36). These findings show that MAM is implicated not only in familial AD, but in sporadic AD as well. Also, the localization of both PSI (a component of γ-secretase) and APP (a γ-secretase substrate) in the same compartment explain how Aβ is transported to adjacent mitochondria (Lustbader et al. 2004 Science 304:448), reportedly via a so-called "unique" pathway (Hansson Petersen et al. 2008 Proc. Natl. Acad. Sci. USA 105:13145.), thus providing a solution to the so-called "spatial paradox."

A number of proteins associated either directly with AD - PSI, PS2, APP, ApoE, CD 147 - or indirectly via the other functions are known to be altered in AD - calcium, lipid, ceramide, and glucose metabolism - are enriched in the MAM.

Mutations in PS1 and PS2, rather than reducing MAM-mitochondrial communication, increase it. This tighter link between mitochondria and ER via the MAM explains the altered phospholipid profiles and elevated cholesterol seen in AD, and explains...
not only the elevated Aβ synthesis, but also the inability of mitochondria to get "off" the ER and get "on" to microtubules for subsequent movement away from the cell body. This difficulty can be especially catastrophic in neurons that require mitochondria to move vast distance from the cell body to axons and dendrites in order to maintain normal brain function. Thus, altered MAM function is a cause of the pathogenesis of both familial and sporadic AD.

Example 19: Analysis of PACS2-KO mice

Another protein previously known to play a role in ER-MAM integrity is phosphofurin acidic cluster sorting protein 2 (PACS2). PACS2 controls the apposition of mitochondria with the ER and appears to regulate of ER-mitochondrial communication via the ER-MAM. PACS2 is found predominantly in the perinuclear region of cells (Simmen et al. (2005) EMBO J. 24:717-729). To investigate if mutations in PACS2 can mimic the effects of mutated PSI MEFs from PACS2-knockout mice were examined by double staining of MEFs with MT Red and anti-tubulin (Atkins et al. (2008) J. Biol. Chem. in press:). Double staining showed a marked perinuclear localization of mitochondria in the PACS2-KO cells (Fig. 26). This result is similar to the results observed in FAD<sup>PS</sup> fibroblasts and in PS1-KD cells. They also showed an alteration in mitochondrial morphology wherein many mitochondria were "doughnut" shaped, possibly because they had detached from microtubules, allowing their tips to fuse. These results indicate that PSI behaves like PACS2, and may function with PACS2 in the same pathway.

Mutations in APP and in the presenilin component of the γ-secretase, which processes APP to produce Aβ have been implicated in the etiology of FAD. Localization of PS1 to adherens junctions at the plasma membrane is consistent with its role in APP processing and cell signaling, since it places this component of γ-secretase complex in close proximity to PM-bound substrates, such as APP and Notch (Leissring et al. (2002) Proc. Natl. Acad. Sci. USA 99:4697-4702; Cupers et al. (2001) J. Neurochem. 78:1 168-1178). Thus, the evidence in support of a role for PS1 in amyloid production in the pathogenesis of AD is strong. The finding that PS1 is also enriched in the ER-MAM, and affects the stability of this compartment and the distribution of mitochondria, point to an additional role for presenilins in the pathogenesis of the disease.

Without wishing to be bound by theory, there are several possible roles for ER-MAM-associated PSI. The possible roles described herein are examples and are not meant to be limiting. Other ER-MAM-associated PSI function are also contemplated.
[00513] ER-MAM may be quantitatively the most important source of γ-secretase activity in the cell. Thus, one possibility is that ER-MAM-localized PS1 also functions as part of the γ-secretase complex, but is in a separate pool located in the ER-MAM (Ankarcrona M, Hultenby K (2002) Biochem. Biophys. Res. Commun. 295:766-770; Hansson et al. (2005) J. Neurochem. 92:1010-1020). This possibility can be tested by determining whether all the components of the γ-secretase complex are present in ER-MAM.


[00515] A third possibility is that mutations in ER-MAM-localized PS1 affect localized [Ca2+] microdomains that ultimately affect neurotransmission (Rintoul et al. (2003) J. Neurosci 23:7881-7888). In this scenario, a localization of PS1 in ER-MAM can explain the various defects in Ca2+ homeostasis seen in cells from FAD patients (Ito et al. (1994) Proc. Natl. Acad. Sci. USA 91:534-538), in cell models (Leissring et al. (1999) J. Neurochem. 72:1061-1068; Leissring et al. (1999) J. Biol. Chem. 274:32535-32538), and in mouse models of FADPS1 (Smith et al. (2005) Cell Calcium 38:427-437; Leissring MA, Akbari Y, Fanger CM, Cahalan MD, Mattson MP, LaFerla FM (2000) J. Cell Biol. 149:793-798; Yoo et al. (2000) Neuron 27:561-572; Begley et al. (1999) J. Neurochem. 72:1030-1039; Barrow et al. (2000) Neurobiol. Dis. 7:1 19-126; Schneider et al. (2001) J. Biol. Chem. 276:1 1539-11544; Tu et al. (2006) Cell 126:981-993). This possibility can be tested by measuring [Ca2+] at or near ER, ER-MAM, and mitochondria. A fourth possibility is that mutations in ER-MAM-localized PS1 interfere with anchorage of mitochondria in the synapse or with the attachment of mitochondria to microtubules and/or their subsequent movement along microtubules (Chang DT, Reynolds IJ (2006) Prog. Neurobiol. 80:241-268). These events are mediated by both ER and mitochondrial Ca2+, and mutated PS1 may prevent the delivery or retention mitochondria to appropriate sites within the cell (e.g. synapses). In one scenario, PS1 located in the ER-MAM regulates the machinery that is involved in mitochondrial movement, via a role in maintaining ER-mitochondrial bridges that allow for proper ER-mitochondrial communication, Ca2+ homeostasis, and binding of mitochondria to kinesin and hence to microtubules via, for example, the Ca2+-binding adapter Miro. A high local [Ca2+] can result in binding of Ca2+ to Miro, thereby preventing efficient attachment of...
mitochondria to microtubules. This can account for the perinuclear localization of mitochondria seen in PSI-mutant cells, the increase in the absolute amount of ER-MAM recovered from PSI-mutant cells, and the aberrant perinuclear accumulations of mitochondria in hippocampal regions of patients with FADPS1. This possibility can be tested by quantitating ER-MAM in normal vs. PSI-mutant cells, and by visualizing mitochondrial movement and distribution in normal and PSI mutant cells.

Example 20: Effects on APP

In order to test whether mutations in presenilin affect ER-to-PM trafficking of APP (Cai et al., (2003) J. Biol. Chem. 278:3446-3454), Western blots can be performed to detect both APP and Aβ in ER, ER-MAM, and mitochondria isolated from control and PSI-mutated cells.

Example 21: Presenilin Transgenic Mice

Transgenic mice that overexpress human PSI with both the M146L and M146V mutations are available (Duff et al., (1996) Nature 383:710-713; Begley et al., (1999) J. Neurochem. 72:1030-1039). Mice in which PSI has been knocked out are embryonic lethal (Handler et al., (2000) Presenilin-1 regulates neuronal differentiation during neurogenesis. Development 127:2593-2606), but PS2 KO mice are viable (Steiner et al., (1999) J. Biol. Chem. 274:28669-28673). Also available are conditional PSI knock out mice in which PSI was eliminated selectively in excitatory neurons of the forebrain, beginning at postnatal day 18 (Yu et al., (2001) Neuron 31:713-726). A double-KO mouse in which the conditional loss of PSI is on a PS2-/- background is also available for analysis (Saura et al., (2004) Neuron 42:23-36). With increasing age, the mutant mice develop striking neurodegeneration of the cerebral cortex and worsening impairments of memory similar to that seen in AD patients (Braak E, Braak H (1997) Acta Neuropathol. 93:323-325; Terry et al., (1991) Ann. Neurol. 30:572-580). Cortical neurons can be isolated from these mice and from appropriate controls of various ages and the distribution of mitochondria can be examined by staining with MitoTracker Red and anti-tubulin. ER, ER-MAM, and mitochondria can also be quantitated in these cells. COX and SDH histochemistry can be performed in freshly frozen brain tissue of the transgenic mice to see if there are alterations in respiratory chain function in neuronal cells. Immunohistochemistry to mitochondrial markers, such as TOM20 (a constitutively-expressed outer membrane marker), can indicate whether there is a change in the distribution and/or intensity of immunostain (indicative of altered organelle numbers) vs. controls.
Example 22: Studies of Brain Tissue

The analyses can be extended to a set of autopic tissues from patients with FAD<sup>PS1</sup>, SAD, and controls (Table 4). Initially, these morphological studies can be confined to the different fields of the hippocampal formation (HF), because this region of the paleocortex is invariably affected in both FAD and SAD. The distribution of mitochondria in the different neuronal compartments (perikaryon, dendrites, axons) can be examined to determine (1) whether there are the alterations in distribution of mitochondria observed in fibroblasts also present in neurons in FAD patients with documented mutations in PS1, and (2) whether there are there similar alterations in hippocampal neurons of patients with sporadic AD given the enrichment of ApoE in ER-MAM.

Table 4. Brain Tissues Available for Studies

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Age</th>
<th>Molecular Defect</th>
<th>Time of Brain Removal</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAD</td>
<td>37</td>
<td>PS1 Mutation</td>
<td>6 hours</td>
</tr>
<tr>
<td>FAD</td>
<td>46</td>
<td>PS1 Mutation</td>
<td>10 hours</td>
</tr>
<tr>
<td>FAD</td>
<td>52</td>
<td>PS1 Mutation</td>
<td>12 hours</td>
</tr>
<tr>
<td>FAD</td>
<td>44</td>
<td>PS1 Mutation</td>
<td>18 hours</td>
</tr>
<tr>
<td>SAD</td>
<td>78</td>
<td>Unknown</td>
<td>14 hours</td>
</tr>
<tr>
<td>SAD</td>
<td>82</td>
<td>Unknown</td>
<td>6 hours</td>
</tr>
<tr>
<td>Controls</td>
<td>46-86</td>
<td>None</td>
<td>8-17 hours</td>
</tr>
</tbody>
</table>

Axonal defects, consisting of swellings that accumulated abnormal amounts of motor proteins, organelles, and vesicles, were found not only in transgenic mice bearing APP (K670N, M671L) and PS1 (A246E) mutations but also in the autopic brains of patients with SAD (Stokin et al., (2005) Axonopathy and transport deficits early in the pathogenesis of Alzheimer's disease. *Science* 307:1282-1288). In the mice, these swellings, some of which were filled entirely by mitochondria, preceded amyloid deposition by more than a year (i.e. the swellings were not a response to amyloid) and appeared to be due to impaired kinesin mediated axonal transport (Stokin et al., (2005) *Science* 307:1282-1288). To answer these questions, mitochondria can be studied using specific immunological probes in neurons of the HF from AD patients and controls, similar to studies that previously performed (Bonilla et al, (1999) *Biochim. Biophys. Acta* 1410:171-182). Clustering of mitochondria in the perinuclear region and aggregation of these organelles in the axons can be examined. The amount of ER, ER-MAM, PM, and mitochondria can be quantitated and the differential distribution of presenilin in these compartments can be determined.
Example 23: Correlation with ApoE Status

Because ApoE is a component of ER-MAM, the ApoE allele status can be determined by PCR/RFLP analysis (Sorbi et al., 1994) Neurosci. Lett. 177:100-102) and the genetics can be correlated with the quantitation of ER-MAM and of mitochondrial distribution to determine if the amount of communication between the ER and mitochondria and/or integrity of ER-MAM is different in patients and cells containing one or two ApoE4 alleles as compared to those containing ApoE2 or ApoE3 alleles. Plasmids over-expressing ApoE3 and ApoE4 can be transfected into human 293T cells to determine if there is a differential effect on ER-MAM and mitochondria.

Because human autoptic brain tissue can be used in the analysis, the time delay between death and autopsy can be examined to determine is there is an adverse affect on the ER-MAM localization of presenilin and on the mitochondrial mislocalization phenotype by sacrificing WT and PS1-mutant mice and harvesting brain and other somatic tissues after various time intervals at room temperature, ranging from 30 min to 18 hours (Table 4). For each sample, the amount of ER-MAM can be isolated, and the presence and total amount of presenilin in ER, ER-MAM, PM, and mitochondria can be quantitated by Western blotting. These analyses can indicate which autoptic samples are appropriate for use and whether they represent a good snapshot of what is actually occurring in the patients.

Example 24: Presenilin Complexes in ER-MAM

The role of in the ER-MAM subcompartment may be different than its role as a component of the γ-secretase complex located primarily in the plasma membrane. If so, ER-MAM localized presenilin functions as a solitary protein, or co-operates with partners other than those known to be part of the γ-secretase complex. A combination of blue-native gels, immunoprecipitation, and protein identification techniques can be used to determine whether presenilin interacts with other partners in the ER or the ER-MAM. If such partners are found, the effects of mutations in these presenilin binding partners on ER-MAM-localization can be determined. Given that presenilin in concentrated in the ER-MAM, Western blots of ER, ER-MAM, and mitochondrial fractions can be probed with antibodies to these proteins, as well as with anti-presenilin to determine if other components of the γ-secretase complex - APH1, nicastrin, and PEN2 - are present in this compartment.

If one or more of these proteins are not present in the ER-MAM fraction, presenilin may have a function in ER-MAM different from that elsewhere in the cell.
Alternatively, if all four proteins are in ER-MAM, it can mean that γ-secretase may be present in this compartment (Sato et al., (2007) *J. Biol. Chem.* In press:). Even if the components of the γ-secretase complex are in the ER-MAM, presenilin may still have another role in this compartment. To determine whether detected components of the γ-secretase complex in the ER-MAM are actually part a single complex, Westerns on blots of ER-MAM fractions separated on "blue-native PAGE" gels can be performed (Schagger H, von Jagow G (1991) *Anal. Biochem.* 199:223-231). In this system, large intact multi- subunit complexes can be separated by blue native polyacrylamide gel electrophoresis (BN PAGE) in the first dimension, and the constituents of the complexes can then be resolved by tricine-SDS-PAGE in the second dimension (Klement et al., (1995) *Anal. Biochem.* 231:218-224). Both the first and second dimension gels can be analyzed by Western blot using anti-presenilin antibodies to see if presenilin is a constituent of a higher order complex, and by antibodies to the other components of the γ-secretase complex to see if they too are present. If the four components co-assemble, there can be co-migration of the Western bands for each component in the first dimension (i.e. BN-PAGE), and separation of the lane by SDS-PAGE in the second dimension can reveal the individual components with appropriate antibodies. Westerns of BN gels of the plasma membrane fraction can serve as a positive control for γ-secretase components (Manfredi et al., (2002) *Nat. Genet.* 25:394-399; Ojaimi et al., (2002) *Mol. Biol. Cell* 13:3836-3844). Since PSI-mediated mitochondrial mislocalization was observed initially in primary human fibroblasts, these experiments can be performed initially on ER-MAM isolated from this tissue. However, it may be that the role of ER-MAM-localized presenilin differs in different tissues. For this reason, ER-MAM isolated from liver and brain (both from mouse and human, where available) can also be examined, presenilin may associate with other as-yet-identified partners in ER-MAM; BN-PAGE can be used in this type of search as well. If there are "MAM-specific" presenilin partners on BN-PAGE, separation of a PS1-positive spot in the second dimension can reveal the constituent components of the complex as spots in the lane of unknown identity (seen by Coomassie or silver staining). Separation of a PSI-immunoprecipitated complex on one-dimensional SDS PAGE can achieve the same goal (a related approach can be to label presenilin with an affinity tag [HA, myc, FLAG, or His6] and immunoprecipitate a PS1-containing complex from isolated ER-MAM using an antibody to the affinity tag). The separated polypeptides can be excised from the gel and sequenced, by standard Edman degradation or by mass-spectrometry. Once PS1-associated candidates are identified, their biological relevance can
be tested in a number of ways. Antibodies to a candidate can be used in SDS-PAGE, BN-PAGE, and in immunoprecipitation assays to see if the candidate is (1) concentrated in the ER-MAM and (2) associated with PS1. Knockdown of the candidate mRNA by RNAi can also knock down presenilin protein. A viable knockout mouse for the candidate gene may be available (Consortium TIMK (2007) Cell 128:9-13), which can used in further studies.

More than 30 proteins have been reported to associate with PS1 (Chen Q, Schubert D (2002) Expert Rev. Mol. Med. 4:1-18), and a search for specific PS1-interacting partners in ER-MAM using immunoprecipitation may result in false positives. However, those searches were done on whole cell extracts. Presenilin binding partners can be identified in isolated ER-MAM, which can reduce the frequency of such false positives. Because the association of presenilin in a higher order complex may be weak (i.e. not observable on BN-PAGE), isolated ER-MAM can be crosslinked (e.g. with formaldehyde) to bind presenilin to its partners, solubilize the fraction, and then co-immunoprecipitate with anti-presenilin antibodies. The crosslink can then be removed (e.g. by heating), run SDS-PAGE, and Westerns to detect presenilin can be performed, or bands from the gel can be isolated to identify them by mass spectrometry. The mass-spectrometry approach has the added advantage of allowing determination of the sequence and identity of the cross-linked proteins. This approach may be more useful than indirect methods, such as yeast 2-hybrid technology, because of the reduced rate of false positives. A related approach is to label presenilin with an affinity tag and immunoprecipitate a PS1-containing complex from isolated ER-MAM using an antibody to the tag.

The search for presenilin partners in ER-MAM is more difficult than merely confirming whether a protein is a constituent of a complex, because the unknown partners cannot be deduced by Western blot analysis in the 2nd dimension. BN PAGE using a higher amount of protein on the gels may have to be performed in order to see the partners by Coomassie or silver staining. Since only the ER-MAM fraction can be loaded on the BN-PAGE gels, rather than whole-cell extracts (as is normally done), the proteins of interest can be concentrated more than 20-fold, and specific bands may be visible in the second dimension. Thicker gels that can accommodate enough sample to identify the spots can be run. While Western blotting reveals only the polypeptide of interest, Coomassie/silver staining is nonspecific, and may reveal too many bands, even on an ER-MAM subfraction. Therefore, the analysis can also be performed on formaldehyde-crosslinked proteins, as described herein. As a complementary approach, ER-MAM can be isolated from the brains of
PS1 knock-in mice or from PS1/PS2 double-knock-out mice vs. controls. BN-PAGE gels of KI or dKO vs. control ER-MAM run side-by-side can be performed to reveal those bands in the control that are missing in the mutated samples. Such missing bands can be authentic PS1 partners. Similar complementary analysis can be performed for PS2. Because degradation and/or loss of completely unrelated polypeptides in the absence of presenilin can result in false positives using this approach, BN-PAGE analyses of ER-MAM can be performed from cells in which either wild-type or mutant presenilin has been overexpressed and compared to untransfected control cells. If the partners are not rate limiting for assembly overexpressed presenilin can bring along higher levels of binding partners. These approaches or combinations thereof can be used to identify PS1-interacting proteins in the ER-MAM.

[00531] Example 25: Tracking Mitochondrial Mislocalization

[00532] The effect of presenilin mutations on anterograde and retrograde axonal transport of mitochondria, on retention and accumulation of mitochondria in nerve terminals, and on the dynamics of mitochondrial fusion and fission can be examined. In order to determine the relevance of these observations to AD, these studies can be conducted in primary neuronal cells derived from normal and FADPS1 or FADPS2 mice. The mitochondrial mislocalization phenotype can be due to (1) a reduced ability of mitochondria to move efficiently along microtubules, or (2) a reduced ability of mitochondria to attach to microtubules in the first place (or some combination of the two). To distinguish between these two possibilities, mitochondrial movement can be tracked in PS1-mutated cells, using a mitochondrially targeted photo-activatable GFP ("mitoDendra") and live-cell imaging. Dendra is a monomeric variant of GFP ("dendGFP") that changes from green to red fluorescent states when photoactivated by 488-nm light. Dendra is completely stable at 37°C, its photoconversion from green to red is both irreversible and high photostable, and it is not phototoxic (Gurskaya et al., (2006) Nat. Biotechnol. 24:461-465). For some applications Dendra can be used instead of MitoTracker dyes, as these have several potential limitations due to their effects on mitochondrial membrane potential and oxidation (Buckman et al., (2001) J. Neurosci. Methods 104:165-176).

[00533] A mitochondrial-targeted Dendra construct in a pTurbo vector (mitoDendra) containing a cleavable N-terminal mitochondrial-targeting signal (MTS) derived from subunit VIII of cytochrome c oxidase (Rizzuto et al., (1989) J. Biol. Chem. 264:10595-10600) can be used to target expressed Dendra into the mitochondrial matrix. When transfected into cells, mitoDendra normally fluoresces green. The optimal conditions
to photoactivate mitoDendra at a defined region of interest (ROI) upon 488-nm laser excitation in confocal microscopy. A preliminary observation to detect the cells on the coverslip was performed at 3% mercury lamp intensity and scanning of the sample at 0.5% 488-nm excitation. Under these conditions, neither bleaching nor photoconversion to red was observed. Only 10 laser iterations were required to photoactivate mitoDendra to red and 20-30 to completely remove residual green fluorescence. To determine if the mitochondria are attached to microtubules, mitochondria can be visualized in living cells by colocalizing red mito-Dendra with TubulinTracker Green (a bi-acetylated version of Oregon Green 488 paclitaxel; Molecular Probes T34075). Multiple regions of interest can be defined in a single neuron, which can include one or several mitochondria at different cellular sites. Transport of multiple mitochondria in different neurons can be followed simultaneously and under the same experimental conditions by time-lapse photography, using confocal microphotography. Unique scan settings at each location (brightness, z-stack) can be defined independently. Several transport parameters can be studied, such as change in position, distance covered, and direction (i.e., distance of movement from an arbitrary origin point set at the cell nucleus). Only mitochondria that move unidirectionally for at least 3 consecutive frames are measured. Thus, transient transfection of cells (e.g. fibroblasts from patients; neurons from transgenic mice; cells and neurons stably-transfected with wt and mutated PS1 and with PS1 knockdown constructs) with mitoDendra can allow the movement of mitochondria containing the reporter (as a green signal) to be tracked.

[00534] Individual mitochondria can be converted to red fluorescence to track their movement in the cell body to determine whether they appear in a specified distance downstream in an axon, and how long it took to get there. Alternatively, mitochondria that are already in an axon can be photoconverted mitochondria to ask the same question and distinguish the dynamic behavior of initially perinuclear mitochondria that may not yet have attached to microtubules from that of mitochondria already attached and moving down axons. The mobilization and movement of mitochondria in the synapse/growth cone and the movement and distribution of tubular (i.e. fused) vs. punctate (fissioned) mitochondria can be examined. ATP distribution and presenilin function in hippocampal neurons can also be examined in the context of loss of presenilin function.

[00535] Mitochondrial dynamics (and Ca2+ handling) in neurons under excitatory and non-excitatory conditions can also be examined. Treatment of neurons with glutamate alters mitochondrial shape (from elongated to punctate) and causes a rapid diminution in their
movement (Rintoul et al. (2003) J. Neurosci 23:7881-7888). This effect is mediated by activation of the N-methyl D-aspartate (NMDA) subtype of glutamate receptors and requires the entry of calcium into the cytosol (Rintoul et al. (2003) J. Neurosci 23:7881-7888). Thus, it can be determined whether mitochondrial movement, distribution, and morphology are altered under excitatory and non-excitatory conditions in control vs. PS1-mutant neurons from transgenic mice, using both the mitoDendra constructs to visualize live cells and imaging of mitochondria in fixed cells.

**Example 26: Effect of the presenilin mutation on the interaction of mitochondria with microtubules in vivo and in vitro**

Pathogenic mutations in PS1 that cause FAD are associated with phenotypes involving impaired mitochondrial movement. Indeed, tau, a substrate of GSK3β, is hyperphosphorylated in AD, and overexpressed tau causes mitochondrial clustering and a reduction in mitochondria in the neurites, due to an impairment in plus-end directed organellar transport (Ebneth et al., (1998) J. Cell Biol., 143, 777-794). This possibility supported by (1) the observation that a PS1 mutation in a mouse PS1 knock-in model impairs GSK3P-mediated kinesin-based axonal transport and also increases tau phosphorylation (Pigino (2003) J. Neurosci., 23, 4499-4508) and (2) the finding of axonal defects, consisting of swellings that accumulated abnormal amounts of microtubule-associated and molecular motor proteins, organelles, and vesicles, in transgenic mouse models of AD (Stokin et al., (2005). Science, 307, 1282-1288). More indirect support for the model derives from studies of APP and amyloid Aβ40-42. It has been found that upon overexpression, APP is targeted to mitochondria and impairs organellar function (Anandatheerthavarada et al., (2003) J. Cell. Biol., 161, 41-54).

Mitochondrial movement can be examined along with interaction with microtubules and microtubule-based motors in PS1-ablated neurons focusing on the relationship between PS1, GSK3P, tau, and kinesins. Given confirmation that mitochondrial motility is defective, PS1-associated defects in mitochondrial distribution can be examined to determine if they affect energy mobilization, and the extent to which mitochondrial distribution defects contribute to neuronal dysfunction in PS1-ablated neurons.

The finding that presenilin is an ER-MAM-associated protein takes AD research in a new direction and provides new approaches to the treatment of familial and sporadic AD.
Example 27: Mitochondria distributed in neurons bearing normal and mutated PS1

Observations in cultured fibroblasts and neurons can be investigated in the clinical situation. (a) Mitochondrial distribution and morphology can be examined in neuronal cells and tissues from normals and from patients and transgenic mice harboring mutations in PS1. (b) Using mitochondrially-targeted photoactivable fluorescent probes ("mitoDendra") and live-cell imaging of neuronal cells, the effect of PS1 mutations on anterograde and retrograde axonal transport of mitochondria, on retention and accumulation of mitochondria in nerve terminals, and on the dynamics of mitochondrial fusion and fission can be examined. In order to determine the relevance of these observations to AD, these studies can be conducted mainly in neuronal cells derived from normal and FAD\textsuperscript{PS1} mice of different ages and under different excitatory states.

Example 28: Role of PS1 in ER-MAM

To address the mechanism of PS1's function in ER-MAM, (a) mitochondrial bioenergetics and redox signaling can be studied in PS1-mutant cells, (b) Ca\textsuperscript{2+} homeostasis in PS1- mutant cells can be analyzed using Ca\textsuperscript{2+} sensitive GFPs ("pericams"), (c) mitochondrial dynamics, neuronal transmission, and Ca\textsuperscript{2+} homeostasis can be examined after disrupting ER-mitochondrial interactions genetically in PACS2-KO mice, and (d) the role of PS1 in maintaining ER-MAM function can be assessed.

Example 29: PS1 and ER-MAM-specific protein partners

The mechanism by which PS1 is enriched in ER-MAM PS1 can be examined to determine if it interacts with other partners in the ER or ER-MAM subcompartments (using blue-native gels, immunoprecipitation, and protein identification techniques), the effects of mutations in PS1 binding partners on ER-MAM localization can be determined.

Example 30: Cells and Tissue Analysis

Cells and/or tissues from one or more of the following sources can be used in connection with the methods described herein. The specific reagent(s) to be analyzed can depend on the analytical approach employed, based on the suitability of the model for analysis. Relevant control cells/tissues suitable for use with the methods described herein
include, but not limited to, cells and tissues from human AD patients, skin fibroblasts from FAD<sup>PSI</sup> and SAD patients, autopic brain from FAD<sup>PSI</sup> and SAD patients, cells and tissues from presenilin-mutant mice, transgenic mice expressing mutant human PSI on a WT mouse background (PSI-Tg) (Duff et al. (1996) Nature 383:710-713), MEFs from knockout mice lacking PSI (PSI-KO) (Donoviel et al. (1999)Genes Dev. 13:2801-2810), MEFs from knockout mice lacking both PSI and PS2 (PSI/PS2-dKO) (Donoviel et al. (1999)Genes Dev. 13:2801-2810), MEFs from PSI/PS2-dKO mice expressing human WT PSI (Donoviel et al. (1999)Genes Dev. 13:2801-2810), MEFs from PSI/PS2-dKO mice expressing human WT PS2 (Donoviel et al. (1999)Genes Dev. 13:2801-2810), mice in which PSI has been ablated conditionally in the forebrain of WT mice (PSI-cKO) (Yu et al. (2001) Neuron 31:713-726), mice in which PSI has been ablated conditionally in the forebrain of PS2-KO mice (PSI/PS2-dKO) (Chen et al. (2008) J. Neurosci. Res. 86:1615-1625), frozen brain from PSI/PS2-dKO mice (Saura et al. (2004) Neuron 42:23-36; Chen et al. (2008) J. Neurosci. Res. 86:1615-1625), cells in which from PSI expression has been knocked down by shRNA, PSI-KD 3T3 cells and CCL131 mouse neuroblastoma cells differentiated with retinoic acid, cells and tissues from PACS2-mutant mice, frozen brain, and MEFs from PACS2-KO mice (Myhill et al. (2008) Mol. Biol. Cell in press), and PACS2-knockdown cells by RNAi (Simmen et al. (2005) EMBO J. 24:717-729).

**Example 31: Mitochondrial Distribution in Neurons Bearing Normal and Mutated PSI**

Mitochondrial distribution and morphology in cells and tissues from normal and FAD<sup>PSI</sup> patients and transgenic mice can be studied, and mitochondrial dynamics can be studied by live-cell imaging.

**Example 32: Analysis of mitochondrial distribution and morphology**

The phenotype of mitochondrial mislocalization observed in FAD<sup>PSI</sup> fibroblasts and in the hippocampus of an FAD<sup>PSI</sup> patient indicate that PSI plays a role in determining mitochondrial distribution, which may be relevant to the pathogenesis of FAD<sup>PSI</sup>. PSI is also present in ER-MAM in brain tissue, the effects observed in somatic cells (e.g. fibroblasts; PSI -knockdown cells) can be investigated in brain and in neuron. These tissues may be more clinically relevant in some aspects.

**Example 33: Analysis of other mutations**
As described herein, studies were performed in fibroblasts isolated from FAD<sup>PS1</sup> patients with the A246E and M146L mutations. Fibroblasts from FAD patients with other PS1 mutations (lines EB [G209V], GF [I143T], WA [L418F]), and WL [H163R]), can be studied using methods including, but not limited to those methods described herein. A fibroblast line carrying a PS2 mutation (line DD [N141I]) and a line carrying a pathogenic mutation in APP can also be examined. In addition to examining ER-MAM in these cells, mutations in PS1 can be examined for their effect on ER-to-PM trafficking of APP (Cai et al. (2003) J. Biol. Chem. 278:3446-3454). Western blotting can be performed to detect both APP and Aβ (and the ratio of Aβ42:Aβ40) in the various subcellular fractions isolated from control and PS1-mutant cells.

Example 34: Transgenic mice that overexpress human PS1 (M146L and M146V mutations)

Mice in which PS1 has been knocked out are embryonic lethals (Handler et al. J (2000) Development 127:2593-2606), but PS2 KO mice are viable (Steiner et al. (1999) J. Biol. Chem. 274:28669-28673). Viable conditional PS1 knock-out mice in which PS1 was eliminated selectively in excitatory neurons of the forebrain, beginning at postnatal day 18 (Yu et al. (2001) Neuron 31:713-726) can be examined. A double-KO mouse in which the conditional loss of PS1 is on a PS2-null background (Yu et al. (2001) Neuron 31:713-726) as well as cells from a second, similar, dKO line (Saura et al. (2004) Neuron 42:23-36) can also be examined. PACS2-KO mice (Atkins et al. (2008) J. Biol. Chem. in press;) from which neurons can also be obtained are also available.

Cortical neurons can be isolated from these mice and from appropriate controls and look at the distribution of mitochondria by staining with MT Red and anti-tubulin. ER, ER-MAM, and mitochondria in these cells can be quantitated. COX and SDH histochemistry can be performed in freshly-frozen brain tissue from the transgenic mice to determine if there are alterations in respiratory chain function in neuronal cells. Immunohistochemistry to mitochondrial markers, such as TOM20 (a constitutively expressed outer membrane marker), can indicate whether there is a change in the distribution and/or intensity of immunostain (indicative of altered organelle numbers) vs. controls.

Example 35: Studies of brain tissue

As described herein, alterations in mitochondrial morphology in the hippocampal formation of a single patient with FAD<sup>PS1</sup> have been observed. These analyses
can be extended to a larger set of autopic tissues from patients with FAD\textsuperscript{PS\textsuperscript{1}}, SAD, and controls. These morphological studies can be performed on the different fields of the hippocampal formation (HF), which is invariably affected in both FAD and SAD. The distribution of mitochondria in the different neuronal compartments (perikaryon, dendrites, axons) can be investigated to determine if: (1) the alterations in distribution of mitochondria observed in fibroblasts are also present in neurons of the HF in FAD patients with documented mutations in PS\textsuperscript{1} (2) there are similar alterations in hippocampal neurons of patients with sporadic AD. In this regard, axonal defects, consisting of swellings that accumulated abnormal amounts of motor proteins, organelles, and vesicles, were found not only in transgenic mice bearing APP (K670N, M671L) and PS\textsuperscript{1} (A246E) mutations but also in the autopic brains of patients with SAD (Stokin et al. (2005) Science 307:1282-1288). In the mice, these swellings, some of which were filled entirely by mitochondria, preceded amyloid deposition by more than a year (i.e. the swellings were not a response to amyloid) and appeared to be due to impaired kinesin-mediated axonal transport (Stokin et al. (2005) Science 307:1282-1288). Mitochondria can be studied using specific immunological probes in neurons of the HF from AD patients and controls (Bonilla et al. (1999) Biochim. Biophys. Acta 1410:171-182) to look for clustering of mitochondria in the perinuclear region and for aggregation of these organelles in the axons. The amount of ER, ER-MAM, PM, and mitochondria can be quantitated and the differential distribution of PS\textsuperscript{1} in these compartments can be determined. COX and SDH histochemistry can be performed on frozen tissue (as opposed to tissue fixed in formalin or paraffin), as described herein. Similar analyses on brain tissue from the M146L/V transgenic mice, the dKO mice, and appropriate controls can also be performed. Since mitochondrial morphology is altered, the expression of mitochondrial fission and fusion proteins (e.g. MFN1/2, FIS1, OPA1, DRP1) in PS\textsuperscript{1}-mutant cells and tissues can be studied by Western blot analysis.

[00559] To determine whether the time delay between death and autopsy has an adverse affect on the ER-MAM localization of PS\textsuperscript{1} and on the mitochondrial mislocalization phenotype, WT and PS\textsuperscript{1}-mutant mice can be sacrificed and brain and other somatic tissues can be harvested after various time intervals at room temperature, ranging from 30 min to 18 hours. For each sample, the amount of ER-MAM can be quantitated, and the presence and total amount of PS\textsuperscript{1} in ER, ER-MAM, PM, and mitochondria can be determined by Western blotting. These analyses can indicate which autopic samples are appropriate for use and
indicate which autoptic tissues represent a good snapshot of what is actually occurring in the patients.

[00560] Example 36: Culturing of explanted primary mouse neurons

[00561] Culturing of explanted primary mouse neurons can be performed based on procedures already described (Friedman et al. (1993) Exp. Neurol. 119:72-78) that yield a relatively pure culture of neurons. To ensure that this is the case, immunostaining for a -internexin, an intermediate filament protein expressed by differentiated postmitotic neurons of the developing CNS, but not by neuroblasts or cells of glial lineage, can be performed (Fliegner et al. (1994) J. Comp. Neurol. 342:161-173).

[00562] Example 37: Visualization of mitochondria, ER, and the cytoskeleton by confocal microscopy

[00563] Fibroblasts are first stained with MT Red and anti-tubulin antibody. A z-series (interval set to 1.4 μm to give non-overlapping sections) of images covering the total cell thickness is collected with a Zeiss LSM510 confocal microscope using a Plan-Neofluar, 0.9 NA objective lens. The pinhole is set to give an optical section of 1.4 μm. Excitation is at 488 nm (for green) and 543 nm (for red). This work can be done in the Imaging Core. Quantitation of mitochondrial distribution in cells. Confocal imaging z sections can be projected into a single image. An area between the nucleus and the cell periphery, as determined by microtubule staining, can be outlined, and the midpoint between the nucleus and the farthest point at the cell periphery can be determined. Using the midpoint, the outlined area can then divided into two parts: regions proximal (A) and distal (B) to the nucleus. Mean grayness values of the MT Red stain can be recorded for the proximal and distal parts. For quantification of mitochondria in the outer edges of a cell, the grayness value for the distal part can be divided by the grayness value for the total area (proximal + distal). Calculation of grayness value for the total area = ([GraynessA x AreaA] + [GraynessB x AreaB])/(AreaA + AreaB).

[00564] Example 38: Immunohistochemistry in brain

[00565] Immunohistochemistry in the brain can be performed on 10μm-thick paraffin-embedded sections using the ABC method or by double-labeling methods with different fluorochromes (Tanji K, Bonilla E (2001) Methods Cell Biol. 65:31 1-332). Polyclonal antibodies against human COX II, ND1, ATPase 8, the iron-sulfur (FeS) protein
subunit of Complex III, and monoclonal and polyclonal antibodies against TOM20 can be used. For neuronal probes, commercially available monoclonal antibodies against MAP2, a perikaryon and dendritic marker, and monoclonal antibodies against MAP5, a marker for neuronal axons can be used. Additional sections can be stained with H-E for conventional microscopic study, with thioflavine S for localization of amyloid deposits, and with a modified Bielschowsky silver stain for evaluation of plaques and neurofibrillary tangles. The samples can be examined with an Olympus BX52 microscope equipped with deconvolution and 3-D reconstruction softwares. Other methods (e.g. COX and SDH histochemistry) can also be used.

**Example 39: Mitochondrial movement in neurons by live-cell imaging**

Mutations in PS1 affect the movement and/or localization of mitochondria in fibroblasts from FADPS1 patients, in COS7 cells transfected with mutated PS1, and in PS1-knockdown 3T3 and CCL131 neuroblastoma cells. Similar analysis can be performed in neurons, which are the clinically relevant tissue in FAD.

The effect of PS1 mutations on anterograde and retrograde axonal transport of mitochondria, on retention and accumulation of mitochondria in nerve terminals, and on the dynamics of mitochondrial fusion and fission can be analyzed. These studies can be conducted in primary neuronal cells derived from normal and FADPS1 mice of different ages and under different excitatory states. The mitochondrial mislocalization phenotype can be due to either (1) a reduced ability of mitochondria to move efficiently along microtubules, or (2) a reduced ability of mitochondria to attach to microtubules in the first place (or some combination of the two). To distinguish between these two possibilities, mitochondrial movement in PS1-mutated cells can be tracked using mitochondrially-targeted photoactivatable GFP ("mitoDendra") and live-cell imaging. Dendra is a monomeric variant of GFP ("dendGFP") that changes from green to red fluorescent states when photoactivated by 488-nm light. Dendra is completely stable at 37°C, its photoconversion from green to red is both irreversible and high photostable, and it is not phototoxic (Gurskaya et al. (2006) Nat. Biotechnol. 24:461-465). For some applications Dendra can be used in place of MitoTracker dyes, as these have several potential limitations due to their effects on mitochondrial membrane potential and oxidation (Buckman et al. (2001) J. Neurosci. Methods 104:165-176).
To determine whether the mitochondria are attached to microtubules in living cells, colocalization of red mito-Dendra with TubulinTracker Green (a bi-acetylated version of Oregon Green 488 paclitaxel; Molecular Probes T34075) can be examined. Multiple regions of interest can be defined in a single neuron, which can include one or several mitochondria at different cellular sites. Transport of multiple mitochondria in different neurons can be followed simultaneously and under the same experimental conditions by time-lapse confocal microphotography. Unique scan settings at each location (brightness, z-stack) can be defined independently. Several transport parameters can be studied, such as change in position, distance covered, and direction (i.e., distance of movement from an arbitrary origin point set at the cell nucleus). In one embodiment, only mitochondria that move unidirectionally for at least 3 consecutive frames are measured. Thus, transient transfection of cells (e.g. fibroblasts from patients; neurons from transgenic mice; neuroblastoma cells and neurons stably-transfected with wt and mutated PS1 and with PS1-knockdown constructs) with mitoDendra can allow tracking of the movement of all mitochondria containing the reporter, as a green signal. In addition, photoconversion of individual mitochondria to red fluorescence can be used to track their movement. Individual mitochondria can be illuminated in the cell body to determine whether they appear in a specified distance downstream in an axon, and how long it took to get there.

Alternatively, mitochondria that are already in an axon can be photoconverted to ask the same question. In this way, the dynamic behavior of initially perinuclear mitochondria that may not yet have attached to microtubules can be compared to that of mitochondria already attached and moving down axons. The mobilization and movement of mitochondria in the synapse/growth cone can be examined using the techniques described herein.

Mitochondrial dynamics (and Ca2+ handling) in neurons under excitatory and non-excitatory conditions can also be examined. Treatment of neurons with glutamate alters mitochondrial shape (from elongated to punctate) and causes a rapid diminution in their movement (Rintoul et al. (2003) J. Neurosci 23:7881-7888). This effect is mediated by activation of the N-methyl D-aspartate (NMDA) subtype of glutamate receptors and requires the entry of calcium into the cytosol (Rintoul et al. (2003) J. Neurosci 23:7881-7888). Thus, both the mitoDendra constructs (to visualize live cells) and imaging of mitochondria in fixed cells can be used to determine whether mitochondrial movement, distribution, and morphology are altered under excitatory and non-excitatory conditions in control vs. PS1-
mutant neurons from transgenic mice. Glutamate induction of synaptic plasticity is age-dependent, that is, explanted rat neurons that are -18 days in vitro (DIV) behaved differently than did "younger ones (-10 DIV)" (Sapoznik et al. (2006) Learn. Mem. 13:719-727). Thus, the various assays can be performed on explanted mouse neurons (described herein) at different DIV.

[00572] Example 40: Transfection of mitoDendra

[00573] Transfection of mitoDendra into neurons can be performed as previously described (Nikolic et al. (1996) Genes Dev. 10:816-825; Ackerley et al. (2000) J. Cell Biol. 150:165-176) using Lipofectamine 2000 or the Promega profection mammalian transfection system. Typically, 10% of the cells are transfected, which provides a sufficient number of cells to allow for multiple measurements. However, to improve gene expression efficiency and to minimize non-specific toxicity derived from transfection approaches, an adenoviral vector mitoDendra construct can also be used into an adenoviral vector (Suhara et al. (2003) Neurobiol. Aging 24:437-451; Magrane et al. (2006) Exp. Cell Res. 312:996-1010; Magrane et al. (2005) J. Neurosci. 25: 10960-10969). In one embodiment, neurons can be imaged 36 hr after transfection.

[00574] Example 41: Neuronal excitation

[00575] The protocol of Rintoul et al. can be used to examine neuronal excitation (Rintoul et al. (2003) J. Neurosci 23:7881-7888). Explanted neurons transfected with mitoDendra can be treated with 30 mM glutamate plus 1 mM glycine for 5 min as described (Rintoul et al. (2003) J. Neurosci 23:7881-7888), in the presence and absence of 5 mM MK801 (which blocks the effect of glutamate), and mitochondrial movement can be monitored. Similar experiments can be performed using 100 mM NMDA plus 1 mM glycine. Other controls can include monitoring movement in the presence of kainite (which depolarizes the plasma membrane) and the calcium ionophore 4-Br-A23187 (Sigma). Depolarization of mitochondrial with, for example, FCCP (carbonyl cyanide 4-[trifluoromethoxy]phenylhydrazone), can be used to examine show the role of mitochondrial ATP synthesis on these processes.

[00576] Example 42: Role of PS1 in ER-MAM

[00577] The effect of PS1 mutations on mitochondrial bioenergetics can be assessed. How Ca2+ homeostasis is altered in PS1-mutated cells can be determined. The effect of disrupting ER-mitochondrial interactions on mitochondrial bioenergetics, on Ca2+
homeostasis, on the subcellular distribution of mitochondria, and on neuronal transmission can be examined. The effect of PSI mutations on ER-MAM function can be examined.

[00578] Example 43: Analysis of calcium homeostasis in normal and PS1-mutated cells

[00579] The results described herein show a defect in ER-mitochondrial calcium trafficking in PS1-KD CCL131 neuroblastoma cells. Owing to its enrichment in ER-MAM, mutations in PS1 alter Ca2+ trafficking, not only between the ER and mitochondria, but also in other regions of the cell. It is known that organellar trafficking is known to take place through a low-affinity Ca2+ uniporter and through an electroneutral Ca2+/Na+-H+ antiporter (Pozzan et al. (1994) Physiol. Rev. 74:595-636), that mitochondrial Ca2+ uptake influences the kinetics and distribution of the cytoplasmic concentration of Ca2+ ([Ca2+]c) (Herrington et al. (1996) Neuron 16:219-228), and that PS1 plays a role in this trafficking (e.g. Tu et al. (2006) Cell 126:981-993)). This trafficking can be monitored using fluorescent Ca2+ reporters targeted to appropriate locations in the cell.

[00580] Alterations in Ca2+ homeostasis in both cellular and animal models of FADPS1 can be assessed using GFP-based calcium reporters ("pericams") targeted to mitochondria and the cytosol. Pericams belong to a class of chimeric probes (Filippin et al. (2005) Cell Calcium 37:129-136) in which GFP derivatives (e.g. yellow YFP) are fused with a Ca2+ binding protein, such as calmodulin (CaM). In pericams, the linear sequence of YFP is cleaved, generating new N- and C-terminals, while the original N- and C-terminals are fused together (i.e. circular permutation). The linkage of CaM and the CaM-binding domain of myosin light chain kinase (ML 3) to the new N- and C-terminals makes pericams sensitive to calcium (Pinton et al. (2007) Meth. Cell Biol. 80:297-325). They have a high affinity for Ca2+ (Kd -0.7 μM), which is favorable for sensing physiological Ca2+ changes. A "ratiometric pericam" has also been developed with an excitation wavelength that changes in a Ca2+-dependent manner (Nagai et al. (2001) Proc. Natl. Acad. Sci. USA 98:3197-3202).

[00581] By targeting a pericam to mitochondria while measuring cytosolic calcium with fura-2, ratiometric data that allows quantitation of the amount of Ca2+ in both compartments. The [Ca2+]c can be quantitated spectrophotometrically but can also be visualized morphologically (see Fig. 25). As with the mitoDendra constructs, the pericam constructs can be inserted into adenoviral vectors to increase the efficiency of transfecting pericams into neurons. Initial transfections with pericams can be done in: (1) PSI/PS2-dKO
MEFs and PS1-KD 3T3 cells; (2) PSI/PS2-dKO MEFs rescued with wild type or FAD<sub>PS1</sub>-mutant PSI; (3) neurons expressing WT or mutated PSI maintained under excitatory vs. non-excitatory states; and (4) mitochondria isolated from WT and dKO MEFs and PS1-KD 3T3 cells. The following protocols can be employed: (1) Simultaneous imaging of [Ca<sup>2+</sup>]<sub>c</sub> (with fura2 or with a nuclear-pericam) and [Ca<sup>2+</sup>]<sub>m</sub> (with a mito-pericam, inverse or ratiometric) in intact cells, followed by sequential treatment with IP3-linked agonists, Tg, and back addition of extracellular Ca<sup>2+</sup>. This protocol allows for quantitation of the [Ca<sup>2+</sup>]<sub>c</sub> and [Ca<sup>2+</sup>]<sub>m</sub> rise evoked by IP3-mediated and residual ER Ca<sup>2+</sup> mobilization and by store-operated Ca<sup>2+</sup> entry. When using pericams to measure [Ca<sup>2+</sup>]<sub>c</sub>, it is targeted to the nucleus. [Ca<sup>2+</sup>]<sub>n</sub> is used as a surrogate of [Ca<sup>2+</sup>]<sub>c</sub>, because it is not feasible use pericams to monitor simultaneously [Ca<sup>2+</sup>]<sub>c</sub> with [Ca<sup>2+</sup>]<sub>m</sub> (Yi et al. (2004) J. Cell Biol. 167:661-672; Csordas G, Hajnoczky G (2001) Cell Calcium 29:249-262). Thus, by measuring separately the extranuclear and nuclear areas, [Ca<sup>2+</sup>]<sub>m</sub> can be determined simultaneously with [Ca<sup>2+</sup>]<sub>n</sub>. (2) Repeat of (1) in cells injected with Ru360 or treated with FCCP to prevent mitochondrial Ca<sup>2+</sup> uptake. This protocol tests the role of mitochondrial Ca<sup>2+</sup> sequestration in the [Ca<sup>2+</sup>]<sub>c</sub> signal in both control and PS mutant cells and is useful to test the dependence of the mito-pericam signal on the ΔΨ<sub>m</sub> and uniporter activity. (3) Simultaneous imaging of [Ca<sup>2+</sup>]<sub>c</sub> (fura2 or rhod2) and [Ca<sup>2+</sup>]<sub>m</sub> in permeabilized cells and in isolated mitochondria treated with IP3, and CaCl<sub>2</sub>. In parallel measurements, ΔΨ<sub>m</sub> can also be monitored. This protocol allows for direct stimulation of the IP3R and allows for comparison of the [Ca<sup>2+</sup>]<sub>m</sub> elevations evoked by IP3- induced Ca<sup>2+</sup> release and by elevation of the bulk cytoplasmic Ca<sup>2+</sup>. The latter can clarify whether the IP3R mitochondrial Ca<sup>2+</sup> transfer or the mitochondrial Ca<sup>2+</sup> uptake mechanism was altered. (4) Repeat protocol (3) in cells incubated in the presence of an EGTA/Ca<sup>2+</sup> buffer (200 µM and 20 µM, respectively), to prevent the IP3-induced [Ca<sup>2+</sup>]<sub>c</sub> rise while monitoring [Ca<sup>2+</sup>]<sub>m</sub>. This protocol specifically tests the local Ca<sup>2+</sup> transfer between IP3R and mitochondria. (5) Measurement of the perimitochondrial [Ca<sup>2+</sup>] using a mitochondrial outer membrane targeted (TOM20) pericam construct. (6) Imaging of [Ca<sup>2+</sup>]<sub>m</sub> and perimitochondrial [Ca<sup>2+</sup>] at the level of single mitochondria in various subcellular regions (perinuclear and peripheral) corresponding to the altered mitochondrial distribution in PS1 -mutant cells. (7) Imaging of Ca<sup>2+</sup> under conditions of neuronal excitation. [Ca<sup>2+</sup>] can be monitored at different locations in normal neurons (e.g. cell body, axons at various distances from the cell body, synapses, dendrites). By combining parallel measurements of Ca<sup>2+</sup> (with pericams) with the assessment of mitochondrial
movement and morphology (with mitoDendra), under both excitatory and nonexcitatory conditions, the "calcium hypothesis" for the pathogenesis of AD can be examined in a highly focused way.

[00582] Measuring [Ca2+]c in the "bulk" cytosol may underestimate the degree of alteration in Ca2+ homeostasis due to a change in [Ca2+] movement between the ER and mitochondria through the ER-MAM. There may be [Ca2+]c "microdomains" located at or near the ER-MAM that reflect changes in Ca2+ homeostasis in a biologically meaningful way but that cannot be detected in the "bulk" cytosol. Accordingly, new pericam constructs that are targeted to other compartments of the cell can be generated. A pericam targeted to the ER or to ER-MAM can be used to measure alterations in [Ca2+] in these compartments. If PSI affects the bridges, changes in [Ca2+] in the ER-MAM of PSI-mutated cells using a "MAM-pericam" can be observed (e.g. fusing the pericam to FAACL4). In one embodiment, "PSI-pericam" is not used because PSI is targeted to other compartments of the cell, such as the plasma membrane. One comparison in such an experiment can be to target a ER-MAM-pericam to PACS2-KD cells (Simmen et al. (2005) EMBO J. 24:717-729) and KO mice (Atkins et al. (2008) J. Biol. Chem. in press. An alteration in [Ca2+] in both PSI- and PACS2-mutant cells can indicate that both proteins are involved in building or maintaining ER-mitochondrial bridges, whereas different [Ca2+] values can indicate that both proteins are not involved in building or maintaining ER-mitochondrial bridges. Mitochondrial [Ca2+]m can be measured using a pericam targeted to the mitochondrial matrix. Given that local Ca2+ concentrations in the vicinity of Miro (which is anchored in the mitochondrial outer membrane [MOM]) affect the attachment of mitochondria to microtubules (via the kinesin adaptor Milton), [Ca2+]m can be measured not only in the mitochondrial matrix, but at the outer membrane as well. Accordingly, a MOM-targeted pericam can be generated by fusing the pericam to either TOM20, a MOM localized component of the mitochondrial importation machinery, or by fusing the pericam to Miro itself (if such a construct does not affect Miro's function). In this way, [Ca2+] can be measured in the actual vicinity of the MOM where the attachment of mitochondria to microtubules takes place. The various pericams can be transfected into control and PSI-mutant cells and the ratio of [Ca2+]c:[Ca2+]m(MAT), [Ca2+]c:[Ca2+]MAM, and [Ca2+]c:[Ca2+]m(MOM) can be determined. If mutated PSI causes haploinsufficiency, the Ca2+ homeostasis defect can be rescued by overexpressing wt-PSI into the cells. Similar experiments can be done in neurons and other cells from the mice.

[00583] Example 44: [Ca2+] assays under neuronal excitation
To define Ca2+ homeostasis in response to extracellular Ca2+ entry, 1 mM glutamate (Eggett et al. (2000) J. Neurochem. 74:1895-1902) can be administered. As a control glutamate with the addition of kynurenic acid, a non-specific glutamate receptor antagonist can be used. Glutamate stimulation with and without the addition of specific blockers (RU360; EMD Biosciences) or release (CGP37157; Tocris Cookson) (Brini et al. (1999) Nature Med. 5:951-954) of mitochondrial Ca2+ uptake can also be performed. In undifferentiated cells that do not express glutamate receptors, a short-term cytosolic Ca2+ peak can be attained with thapsigargin (1 μM; Sigma). Because of the variability in the number of cells that respond to glutamate stimulation and because amplitude and delay of the Ca2+ response may vary from cell to cell, more than one cell line may have to be analyzed in order to obtain statistically significant measurements (Eggett et al. (2000) J. Neurochem. 74:1895-1902). Alternatively, intracellular Ca2+ spikes can be generated by stimulation of P2X ion channels, which respond to micromolar concentrations of extracellular ATP (North RA (2002) Physiol. Rev. 82:1013-1067). This approach allows for the depolarization of a large number of cells irrespective of their state of differentiation. Analysis of variance can also be performed to compare the various cell lines. If the data are not normally distributed, either the Kruskal-Wallis or Mann-Whitney U tests can be utilized.

Example 45: Analyses of PACS2-KO cells in which ER-MAM communication is disrupted

PACS2 is a protein adaptor that controls ER-mitochondria contacts (Simmen et al. (2005) EMBO J. 24:717-729). Experimental disruption of the physical communication between the ER and mitochondria in PACS2-KO mice can mimic the many of the various phenotype seen in PS1-mutated cells, thereby indicating the role of PS1 (and PACS2) in ER mitochondrial communication and the pathogenesis of FAD<sup>PS1</sup>. Loss of ER-MAM function - whether via mutated PS1 or mutated PACS2 - can indeed be relevant to the pathogenesis of FAD<sup>PS1</sup>.

The subcellular distribution of ER-MAM and of PS1, and the effects of altering ER-mitochondrial communication on neuronal transmission and on calcium homeostasis can be examined in normal and PS1-mutated mouse neurons using PACS2-KO mice.

Two types of experiments with PACS2-KO mice and MEFs can be performed using procedures described herein. (1) "Static" experiments in isolated cells and
tissues. This can include (a) examination of mitochondrial morphology in fixed cells (using MT Red and antibodies to tubulin, PS1, and other relevant markers); (b) quantitation of the amount of ER, ER-MAM, PM, and mitochondria; (c) analysis of ER-MAM function; (d) analysis of mitochondrial bioenergetics; (e) determination of the distribution of PS1 (and other relevant markers, including APP and Aβ) in these compartments; (f) analysis of mitochondrial distribution in PACS2-KO brains; and (g) analysis of the expression of fusion/fission proteins. (2) "Dynamic" experiments in living cells. This can include (a) monitoring mitochondrial distribution and movement using MT Red and adenoviral-transfected mitoDendra; and (b) monitoring Ca2+ levels in various subcellular compartments using fluorescent Ca2+ reporters. All relevant methods are described herein.

[00589] Example 46: Analysis of the role of PS1 in ER-MAM function

[00590] Because the mitochondrial maldistribution observed in PS1-mutant cells was also observed in PACS2-KO cells, PS1 may play a role in maintaining ER-MAM integrity and effective ER-mitochondrial communication, and defects in ER-MAM function may play a role in the pathogenesis of the disease.

[00591] Antibodies to known ER-MAM components can be used to characterize further the association of PS1 with ER-MAM and the disposition of this compartment in neurons, an unexplored area. ER-MAM can be isolated from WT, PS1-KO, PS1/PS2-dKO, and PACS2-KO brain and the amount of ER-MAM obtained can be quantitated and compared to those obtained in other tissues (e.g., liver, muscle). Measuring the amount of ER-MAM indicates the qualitative nature of the ER-MAM compartment and provides little mechanistic insight into whether PS1 is required for ER-MAM function. Accordingly, such function can be assayed using three different approaches: phosphatidylethanolamine (PE) formation, sensitivity to cinnamycin, and fluorescence resonance energy transfer (FRET) in the ER-MAM. PE formation. The ER-MAM is a locus of phospholipid synthesis. Notably, phosphatidylserine (PS) is transported from the ER-MAM to mitochondria, where it is decarboxylated to form PE; the PE is then retransported back to the ER-MAM, where it is methylated to form phosphatidylcholine (PC) (Achleitner et al. (1999) Eur. J. Biochem. 264:545-553). If ER-MAM function is compromised, the rate of transport of PS from the ER-MAM to the mitochondria is reduced, and hence the production of PE inside of mitochondria is also reduced (Achleitner et al. (1999) Eur. J. Biochem. 264:545-553; Achleitner et al. (1995) J. Biol. Chem. 270:29836-29842; Wu WI, Voelker DR (2001) J. Biol. Chem.
276:71 14-7121; Schumacher et al. (2002) J. Biol. Chem. 277:51033-51042). Consistent with this idea, cholesterol and phospholipids (for example, PE, PS, and PC) were selectively reduced an AD "double-transgenic" mouse model (i.e. mutations in both APP and PS1) (Yao et al. (2008) Nerochem. Res. in press). The conversion of PS to PE can be examined by adding 3H-Ser to WT and mutant cells and measuring the amount of 3H-PE (and 3H-PS) produced as a function of time (Achleitner et al. (1995) J. Biol. Chem. 270:29836-29842).

[00592] Example 47: Sensitivity to cinnamycin

[00593] Cinnamycin, also called Ro 09-0198, is a tetracyclic peptide antibiotic that is used to monitor the transbilayer movement of PE in biological membranes (Choung et al. (1988) Biochim. Biophys. Acta 940:171-179; Choung et al. (1988) Biochim. Biophys. Acta 940:180-187). Cinnamycin binds specifically to PE (Choung et al. (1988) Biochim. Biophys. Acta 940:171-179; Choung et al. (1988) Biochim. Biophys. Acta 940:180-187), and was used in a screen to identify mutants defective in PS transport through the ER-MAM (Emoto et al. (1999) Proc. Natl. Acad. Sci. USA 96:12400-12405). There is be a minimum inhibitory concentration (MIC) at which cinnamycin binds to PE in normal cells and kills them via cytolysis, whereas cinnamycin at the same concentration will have reduced binding to PE in AD cells, and not kill them. An easy way to distinguish between the two is by a "live-dead" assay (e.g. living cells are green whereas dead cells are red). Thus, the MIC in WT and PS1-mutant cells can be measured.

[00594] Example 48: Fluorescence Resonance Energy Transfer

[00595] Modified from Man et al. (2006) J.Lipid Res. 47: 1928. Diglycerol acyltransferase 2 (DGAT2) and stearoyl-CoA desaturase 1 (SCD1, also called SCD) are both localized in the MAM and interact with each other in that compartment. Both DGAT2 fused to yellow fluorescent protein (DGAT2-YFP) and SCD1 fused to cyan fluorescent protein (SCD1-CFP) are expressed in cells. In one embodiment, both fusion proteins can be expressed from a bicistronic plasmid. YFP is detected by illuminating the cells at 488 nm and detecting at 560 nm. CFP is detected by illuminating the cells at 403 nm and detecting fluorescence at 470 nm. In cells expressing both YFP and CFP, a FRET can be observed by detecting yellow fluorescence at 560 nm upon illumination in the blue at 403 nm. In control cells co-expressing DGAT2-YFP and SCD1-CFP, this FRET can be observed and the degree of FRET (intensity; number of FRET-positive cells compared to all transfected cells) can serve as a baseline value. The same construct(s) can be transfected in PS1-mutant cells and
the degree of FRET measured and compared to FRET values observed in control cells. If MAM integrity is altered, the kinetics of the apposition of DGAT2-YFP to SCD1-CFP can be increased, thereby causing a increase in FRET intensity and/or in the number of FRET-positive cells. Without being bound by theory, this increase can occur because FRET signal increases with the 6th power of the distance between the YFP and CFP moieties.

[00596] Diacylglycerol-O-acyltransferase 2 (DGAT2) and stearoyl-CoA desaturase 1 (SCD1) - form a dimeric complex in the ER-MAM (Man et al.(2006) J. Lipid Res. 47:1928-1939). By appending YFP to DGAT2 and CFP to SCD1, Man et al. (Man et al.(2006) J. Lipid Res. 47:1928-1939) demonstrated FRET between SCD1-CFP and DGAT2-YFP, indicating that the two proteins are adjacent to each other (within a few nm) in the ER-MAM. Normal cells will have a strong FRET signal, because in ER-MAM membranes having altered structure, DGAT2 and SCD1 can move laterally through the ER-MAM lipid and "find" each other easily. However, in ER-MAM having altered structure from FAD$_{PS1}$ patients, the FRET signal can be altered due to differences in traversing the membrane, and the FRET signal can be increased significantly (the signal falls off with the 6th power of the distance between the two interacting moieties). This increase in FRET can be exploited in a chemical screen to search for compounds that improve the FRET signal (indicative of improved ER-MAM integrity), as a treatment strategy in FAD$_{PS1}$.

[00597] Plasmids encoding SCD1-CFP and DGAT2-YFP (Man et al. (2006) J. Lipid Res. 47:1928-1939) have been verified to be functional (i.e. the YFP 488-nm fluorescence at 560 nm, and CFP 403-nm fluorescence at 470 nm have been detected). FRET can be examined in cells expressing both YFP and CFP by detecting fluorescence at 560 nm upon illumination in the blue at 403 nm. A construct in which both genes are on a bicistronic vector and are expressed stably can also be generated.

[00598] Example 49: Identification of PSI Partners in the ER-MAM

[00599] ER-MAM-localized PSI can function either as a solitary protein, or co-operate with partners other than (or in addition to) those known to be part of the $\gamma$-secretase complex. The pleiotropic effects of mutations in PSI in FAD$_{PS1}$ patients (e.g. altered lipid, glucose, cholesterol, and Ca2+ metabolism) can indicate that PSI functions with one or more partners.

[00600] PSI can be investigated to determine if it interacts with other partners in the ER-MAM. If such partners are found, the effects of mutations in these PSI binding
partners on ER-MAM localization can be determined. Given that PS1 in concentrated in the ER-MAM, and that there is strong γ-secretase enzymatic activity in ER-MAM (Fig. 4), analysis can be performed to determine if the other components of the γ-secretase complex - APH1, nicastrin, and PEN2), as well as the regulatory molecules CD147 and TMP21 - are present in this compartment as well.

[00601] Western blots of subcellular fractions (e.g. ER, ER-MAM, plasma membrane [PM], and mitochondrial fractions) from WT mouse tissues and cells can be probed with antibodies to these proteins, as well as with anti-PS1. As negative controls, Westerns on PS1/PS2 dKO mouse brains and/or PS1-KO MEFs can be performed. If differences are observed in the Western pattern in the ER-MAM fraction compared to other compartments, it can indicate that PS1 may have a function in ER-MAM different from that elsewhere in the cell. Alternatively, finding all the components of the γ-secretase complex in the ER-MAM may still not eliminate the possibility of another role for PS1 in this compartment. To determine whether all detected components of the γ-secretase complex in the ER-MAM are actually part a single complex, Westerns on blots of ER-MAM fractions from test and control (PS1-null) mouse brain can be separated on "blue-native PAGE" gels (Schagger H, von Jagow G (1991) Anal. Biochem. 199:223-231). In this system, large intact multi-subunit complexes can be separated by blue native-polyacrylamide gel electrophoresis (BN PAGE) in the first dimension, and the constituents of the complexes can then be resolved by tricine-SDS-PAGE in the second dimension (Klement et al. (1995) Anal. Biochem. 231:218-224). Both the first and second dimension gels can be analyzed by Western blot using anti-PS1 antibodies to see if PS1 is a constituent of a higher order complex, and by antibodies to the other components of the γ-secretase complex to see if they too are present. If all the subunits co-assemble, there can be co-migration of the Western bands for each component in the first dimension (i.e. BN-PAGE), and separation of the lane by SDS-PAGE in the second dimension can reveal the individual components with appropriate antibodies. Westerns of BNPAGE gels of the plasma membrane fraction can serve as a positive control for authentic γ-secretase components. Since the role of ER-MAM-localized PS1 may differ in different tissues, search can be extended to ER-MAM isolated from liver and brain. BN-PAGE system has been previously characterized (Manfredi et al. (2002) Nat. Genet. 25:394-399; Ojaimi et al. (2002) Mol. Biol. Cell 13:3836-3844). PS1 may associate with other as-yet-unidentified partners in ER-MAM, and BN-PAGE can be used in this type of search as well. However, to isolate PS1-interacting proteins in ER-MAM, a more
direct, two-tiered approach can be performed using: (1) tandem affinity-purification (TAP Tag) in cell culture, and (2) direct IP in lysates from WT and PS1/2-dKO mouse brains and PS1-KO cells.

Example 50: TAP Tag

TAP-tagging is a highly-selective tandem affinity purification method (Rigaut et al. (1999) Nature Biotechnol. 17:1030-1032). In brief, the "bait" gene of interest (i.e. PS1) is fused with two "tandem tags" - a calmodulin binding site followed by an IgG binding domain - with a tobacco etch virus (TEV) cleavage site located between the two tags. The construct is expressed in cells and PS1-associated complexes in the purified ER-MAM fraction are first isolated by its strong binding to IgG resin via the IgG-binding domain of the PS1 fusion protein. After washing, TEV protease is added to release the bound material (i.e. the tagged PS1 complexes). The eluate is then incubated with calmodulin coated beads in the presence of calcium. This second affinity step is required to remove the TEV protease as well as traces of contaminants remaining after the first affinity selection. After washing, the bound material is released with EGTA. The purification is monitored at each step by Western blot analysis. Finally, the candidate proteins are resolved on silver-stained SDS gels and identified by mass spectrometry. The procedure can also be performed using empty vector (negative control) and on plasma membrane fractions (positive control). This method (Rigaut et al. (1999) Nature Biotechnol. 17:1030-1032) has two advantages. First, by purification with two consecutive antibodies, false positives can be minimized, which is a problem in any co-immunoprecipitation-based method for isolating interacting proteins. Second, the method allows for the purification of protein complexes under mild conditions, preserving the interactions among the proteins that form part of the complex to be purified. As a backup approach, the method of Tsai and Carstens (Tsai A, Carstens RP (2006) Nature Protocols 1:2820-2827) in which a 2x Flag tag replaces the calmodulin tag can be used. In this case the Flag tagged PS1 complexes are purified further by binding to beads containing anti-Flag antibodies, which are then released from the beads with Flag peptides.

As described (Tsai A, Carstens RP (2006) Nature Protocols 1:2820-2827), cells can be transfected stably with a bicistronic vector plasmid containing the CMV-derived eukaryotic promoter upstream of PS1 with a downstream IRES sequence followed by an antibiotic selection marker (e.g. puroR or neoR). Isolated ER-MAM (up to 40 mg) can be mixed with IgG beads with gentle rotation for 4-16 h at 4°C. After washing, the bound IgG resin can then be treated with 100 U of TEV protease for 16 h at 4°C to release Flag tagged...
PSI complexes. The complexes containing solution can be separated from the IgG resin with a 1-ml Micro Bio-Spin column. Eluates can be pooled and mixed with anti-Flag resin (Sigma) with gentle rotation at 4°C for 4 h, followed by washing the Flag-PSI complex-bound beads with 1 ml of TBS wash buffer, 3X at 4°C. Finally, Flag-tagged PSI-associated complexes can be eluted from the resin with 3X Flag peptide in TBS buffer. The calmodulin method (Rigaut et al. (1999) Nature Biotechnol. 17:1030-1032) is fundamentally similar (Jorba et al. (2008) J. Gen. Virol. 89:520-524).

**Example 51: Immunoprecipitation (IP)**

In parallel to the TAP method, PSI antibodies that have been proven effective in IP, and the PSI knockout mice and cells can be used. The specific antibodies can be efficient to pull down PSI and its interacting proteins. The ER-MAM from the forebrains of PSI/2-dKO mice, or from cultured blastocysts from PSI-KO mice can be used as negative controls. In this approach, ER-MAM from wild-type and dKO mouse brains (or WT and PSI-KO cells) can be purified as described herein, and anti PSI antibody can be used to pull down PSI and its interacting proteins. Two antibodies that have been tested: PS1-CTF (Sigma) (Serban et al. (2005) J. Biol. Chem. 280:36007-36012) and monoclonal antibody MAB5232 (Chemicon) (Laudon et al. (2005) J. Biol. Chem. in press; Nakaya et al. (2005) J. Biol. Chem. 280:19070-19077) can be used. The co-immunoprecipitated proteins can be separated by SDS-PAGE, and the bands that are unique to the WT lane can be excised and sent for mass spectrometry analysis. With both techniques, once PSI-associated candidates are identified, their biological relevance can be tested in a number of ways. Antibodies to a candidate can be used in SDS-PAGE, BN-PAGE, and in immunoprecipitation assays to see if the candidate is (1) concentrated in the ER-MAM and (2) associated with PSI. Knockdown of the candidate mRNA by RNAi can also knock down PSI protein. A viable knock-out mouse for the candidate gene may be available (Consortium TIMK (2007) Cell 128:9-13), which can be used for further studies. Moreover, if antibodies against the candidate proteins are available, they can be used to reverse-IP PSI from the ER-MAM preparation from WT and PSI-mutant mice/cells. If the antibodies against the candidate proteins are not available, myc-tagged candidates can be generated and a two-directional IP can be performed with the TAP construct of PSI in transfected cell cultures. The same two approaches can be used on other subcellular fractions (e.g. bulk ER; PM) to look for differences in binding partners in different fractions. If differences are found among fractions, this comparison can provide information regarding the function(s) that PSI may play in different parts of the cell (e.g.
cleavage of Notch in the PM vs. a role in mitochondrial distribution and lipid metabolism in the ER-MAM).

**[00607]** Any approach to find protein partners may produce false positives, however the TAP Tag method, which uses two affinity purification steps, minimizes this problem. Moreover, the use of two different but complementary TAP Tag methods - one involving a calmodulin tag, and the other a Flag tag - can yield the same partners with both procedures, and these partners are more likely to be authentic, with the "non overlapping" set of partners more likely to be spurious.

**[00608]** Searches for protein-protein interactions can be conducted on whole cell extracts. PS1 binding partners can be investigated specifically in isolated ER-MAM, which can reduce the frequency of such false positives. Moreover, by enriching for the correct fraction, the chance of finding the true positives is increased simply because there is more protein to start with, and thus less protein is likely to be lost in the washing process.

**[00609]** The issue of false negatives (i.e. failure to identify a partner due to weak interacting proteins) is more difficult to address, as it boils down to a tradeoff between the strength of the washing conditions and the number of proteins recovered. From a practical standpoint, the "strong" positives can be examined first to ensure that they are identified and verified. These new partners can then be used as "bait" in further rounds of TAP Tag to look for new partners that may have been missed the first time around, as proteins that interacted weakly with PS1 may interact strongly with others in the complex.

**[00610]** Example 52: PS radiolabel assay

**[00611]** Uniformly-labeled 3H-Ser can be added to cells for various time intervals (e.g. 0, 1, 2, 4, 6 hours). The cells are killed and the lipids are concentrated by chloroform extraction. The extract is can analyzed by thin layer chromatography to identify various lipids (e.g. phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine, total triglycerides, sphingomyelin) using purified standards (identified by spraying the plate with iodine to reveal the bands/spots) and the 3H label is counted. The 3H data can be plotted vs. time and normalized against any variation of protein content among samples. A reduction in slope for 3H-PE vs. time in test vs. control is be indicative of an ER-MAM transport defect.

**[00612]** Example 53: Localization of Presenilins to ER-MAM
[00613] As described herein, presenilins residing in the MAM are functionally active, acting as the catalytic core of the γ-secretase complex however PS1 and/or PS2 can also be involved in other functions in the MAM compartment. The finding that most of the γ-secretase activity is located in ER-mitochondria connections explains the observation of mitochondrial oxidative damage associated with abnormal APP processing (Atamna et al, Mitochondrion 2007, 7:297-310). Moreover, it explains how Aβ accumulates in mitochondria (Du et al, Nat. Med. 2008, 14:1097-1105), as well as provide the basis for the interaction between PS1 and a number of known mitochondrial proteins.


[00615] Example 54: Alzheimer Disease and Presenilins

[00616] Alzheimer disease (AD) is a neurodegenerative dementing disorder of late onset, with a relatively long course (Mattson MP (2004) Nature 430:631-639) There is progressive neuronal loss, especially in the cortex and the hippocampus. The two main histopathological hallmarks of AD are the accumulation of extracellular neuritic plaques, consisting mainly of β-amyloid (Aβ), and of neurofibrillary tangles, consisting mainly of hyperphosphorylated forms of the microtubule-associated protein tau (Goedert M, Spillantini MG (2006) Science 314:777-781; Roberson et al. (2007) Science 316:750-754).

[00617] The majority of AD is sporadic (SAD), but at least three genes - amyloid precursor protein (APP), presenilin-1 (PS1), and presenilin-2 (PS2) - have been identified in the familial form (FAD). Clinically, FAD due to mutations in PS1/2 is similar to SAD

Hippocampal cultures from transgenic mice expressing the PS1 A246E mutation have depressed evoked synaptic currents, due to reduced synaptic density (Prillier et al. (2007) J. Biol. Chem. 282:1 119-1 127). These results indicate that AD is ultimately a disease of synaptic transmission (Selkoe DJ (2002) Science 298:789-791; Walsh DM, Selkoe DJ (2004) Neuron 44:181-193) wherein the pathogenesis of AD involves a relationship between two or more of amyloid, presenilins, predisposing factors, and other cellular processes.

Example 55: Presenilins Localization


Besides PS1/2, the γ-secretase complex contains five other proteins: APH1, PEN2, nicastrin (NCT, also called APH2) (De Strooper B (2003) Neuron 38:9-12), and two regulatory subunits, CD147 (Zhou et al. (2005) Proc. Natl. Acad. Sci. USA 102:7499-7504) and TMP21 (Chen et al. (2006) Nature 440:1208-1212). Since γ-secretase complexes with different molecular masses and subunit compositions have been found (Gu et al. (2004) J. Biol. Chem. 279:31329-31336), different subunits may affect the localization and/or function of the complex. PS1, APH1, NCT, and PEN2 are found in the plasma membrane (Hansson et al. (2005) J. Neurochem. 92:1010-1020). Using immunoelectron

**Example 56: Mitochondria and mitochondrial movement**


**[00627]** Example 57: The "calcium hypothesis" in FAD

**[00628]** The predominant "amyloid hypothesis" invokes the toxic effects of APP and amyloid in the pathogenesis of AD (Hardy J, Selkoe DJ (2002) Science 297:353-356). The role of calcium in the pathogenesis of AD is more controversial, but there is a growing body of evidence to implicate calcium, at least in FAD due to mutations in PS1 (FADPS1). The overall thrust of the "calcium hypothesis" is that presenilin mutations affect ER Ca2+ signaling (Mattsson et al. (2000) Trends Neurosci. 23:222-229; Smith et al. (2005) Cell Calcium 38:427-437), resulting, in some as-yet undefined way, in neuronal degeneration.

including increased ER Ca2+, a lower threshold for kainic acid-induced glutamate release, and increased glutamate-induced Ca2+ signals (Schneider et al. (2001) J. Biol. Chem. 276:1 1539-1 1544). PS2 associates with sorcin, a Ca2+-binding modulator of the muscle calcium channel/ryanodine receptor (RyR) (Pack-Chung et al. (2000) J. Biol. Chem. 275:14440-14445) that is in close apposition to both ER and mitochondria (Pickel et al. (1997) J. Comp. Neurol. 386:625-634). Presenilins are required for Ca2+ influx into cells from "store operated Ca2+" (SOC) channels located in the plasma membrane ("capacitative calcium entry" [CCE]).


[00631] During CCE, two elements are required to reduce diffusion of Ca2+ into the cytosol in the vicinity of SOC channels: (1) an active sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) and (2) neighboring mitochondria (Jousset et al. (2007) J. Biol. Chem. 282:1 1456-1 1464). In CCE, the mitochondria play two roles: they scavenge remaining Ca2+ that cannot be handled by the SERCA, and they provide local ATP to buffer [Ca2+] (Jousset et al. (2007) J. Biol. Chem. 282:1 1456-1 1464). Both functions require that mitochondria be located in the vicinity of the SOC channels near the narrow and extended ER-PM junctions in "microdomains" linking the SOC channels to the SERCA (Jousset et al. (2007) J. Biol. Chem. 282:1 1456-1 1464). Thus, the subcellular distribution of mitochondria in microdomains is critical not only for providing ATP as a source of oxidative energy for various cellular processes, such as neurotransmission (Hollenbeck PJ, Saxton WM (2005) J. Cell Sci. 118:541-5419; Hollenbeck PJ (2005) Neuron 47:331-333), but is also critical for "non-oxidative" functions, such as Ca2+ homeostasis (Hollenbeck PJ (2005) Neuron 47:331-333; Alonso et al. (2006) Cell Calcium 40:513-525). Thus, mutations in PSI can have devastating effects on neuronal function.
The "amyloid hypothesis" and the "calcium hypothesis" need not be mutually exclusive explanations for the pathogenesis of AD, as connections among PSI, APP, and Ca2+ signaling may actually exist. First, there is evidence that PSI functions as a passive ER Ca2+ leak channel (Nelson et al. (2007) J. Clin. Invest. 117:1230-1239; Tu et al. (2006) Cell 126:981-993) and that expression of FAD-linked PSI mutations disrupt this functionality (Nelson et al. (2007) J. Clin. Invest. 117:1230-1239). γ-secretase-mediated cleavage of APP yields an intracellular fragment, called the APP intracellular domain (AICD), which then translocates to the nucleus (Cupers et al. (2001) J. Neurochem. 78:1 168-1178).

However, AICD also regulates phosphoinositide-mediated Ca2+ signaling in a mechanism involving modulation of ER Ca2+ stores (Leissring et al. (2002) Proc. Natl. Acad. Sci. USA 99:4697-4702); notably, only the AICD fragment of APP has this property (Leissring et al. (2002) Proc. Natl. Acad. Sci. USA 99:4697-4702). Thus, the proteolysis of APP may be required for intracellular Ca2+ signaling, and a defect in such processing in PSI-mutated cells can explain alterations in the pleiotropic effects on Ca2+ handling described herein.

**Example 58: Endoplasmic reticulum - mitochondria-associated membranes (ER-MAM)**

The ER is the cell's main store of calcium, which is released upon stimulation by input signals such as IP3 and sphingosine-1-phosphate (Berridge MJ (2002) Cell Calcium 32:235-249). The main site of calcium uptake is the mitochondrion, but mitochondria are not passive "sinks" - they use calcium actively, for example, to activate dehydrogenases for intermediate metabolism (Robb-Gaspers et al. (1998) EMBO J. 17:4987-5000). Thus, the ER and the mitochondria can be linked not only biochemically but also physically (Jousset et al. (2007) J. Biol. Chem. 282:1 1456-1 1464; Rizzuto et al. (1998) Science 280:1763-1766; Csordas et al. (2006) J. Cell Biol. 174:915-921). In 1993, Cui et al. (Cui et al. (1993) J. Biol. Chem. 268:16655-16663) described the localization of phosphatidylethanolamine N-methyltransferase 2 (PEMT), an enzyme of phospholipid metabolism, in a unique membrane subfraction of the ER that was subsequently termed ER-MAM (Rusinol et al. (1994) J. Biol. Chem. 269:27494-27502). The same compartment was also found in yeast (Gaigg et al. (1995) Biochim. Biophys. Acta 1234:214-220; Prinz et al. (2000) J. Cell Biol. 150:461-474).
Since then, about two dozen proteins have been found to be concentrated in the ER-MAM, most of which are enzymes involved in the metabolism of glucose (e.g. glucose-6-phosphatase [G6PC] (Bionda et al. (2004) Biochem. J. 382:527-533), phospholipids (PEMT; diacylglycerol acyltransferase 2 [DGAT2] (Man et al. (2006) J. Lipid Res. 47:1928-1939), ceramide (ceramide glucosyltransferase [UCGC] (Ardail et al. (2003) Biochem. J. 371:1013-1019), gangliosides (β-galactoside a(2-6) sialyltransferase [SIAT1/ST6GAL1] (Ardail et al. (2003) Biochem. J. 371:1013-1019), cholesterol (sterol Oacyltransferase 1 [SOAT1], also called acyl-coenzyme Axholesterol acyltransferase [ACAT1] (Rusinol et al. (1994) J. Biol. Chem. 269:27494-27502), and fatty acids (stearoyl-CoA desaturase [SCD] (Man et al. (2006) J. Lipid Res. 47:1928-1939); fatty acid-CoA ligase 4 [FACL4] (Lewin et al. (2002) Arch. Biochem. Biophys. 404:263-270), and in lipoprotein transport (microsomal triglyceride transfer protein large subunit [MTTP] (Rusinol et al. (1994) J. Biol. Chem. 269:27494-27502). ER-MAM is a physical bridge that connects the ER to mitochondria (Csordas et al. (2006) J. Cell Biol. 174:915-921). This result explains why it has been almost impossible to subfractionate pure mitochondria uncontaminated by ER (on the other hand, the reverse - the isolation of ER uncontaminated by mitochondria - is relatively easy). Moreover, the IP3 receptor (IP3R), which binds IP3 to stimulate Ca2+ transfer to mitochondria, is also a ER-MAM protein (Csordas et al. (2006) J. Cell Biol. 174:915-921), as is the ryanodine receptor (Hajnoczky et al. (2002) Cell Calcium 32:363-377) and, most recently, the sigma-1 type opioid receptor (SIG1R/OPRS1) (Hayashi T, Su TP (2007) Cell 131:596-610), emphasizing the intimate relationship between ER and mitochondria in regulating calcium.

RyRs interact with, and are regulated by, both PS1 (Rybalchenko et al. (2008) Int. J. Biochem. Cell Biol. 40:84-97) and PS2 (Hayrapetyan et al. (2008) Cell Calcium in press:), and IP3R interacts with PS2 (Cai et al. (2006) J. Biol. Chem. 281:16649-16655). There is a functional coupling between RyRs and mitochondria that allows for "tunneling" of Ca2+ from the ER to mitochondria (Kopach et al. (2008) Cell Calcium 43:469-481). Only one protein has been implicated in the regulation of ER mitochondrial communication via the ER-MAM: phosphofurin acidic cluster sorting protein 2 (PACS2), which controls the apposition of mitochondria with the ER (Simmen et al. (2005) EMBO J. 24:717-729). PACS2 interacts with transient receptor potential protein 2 (TRPP2/PKD2), a Ca2+-permeable cation channel (Kottgen et al. (2005) EMBO J. 24:705-716). PACS2 is found predominantly in the perinuclear region of cells (Simmen et al. (2005) EMBO J. 24:717-729), as is TRPP2.

PACS2 translocates to mitochondria upon stimulation with pro-apoptotic agents such as staurosporin (Simmen et al. (2005) EMBO J. 24:717-729).

[00638] Example 59: Mitochondrial Dynamics in Neurodegenerative Disease

[00639] Mutations in presenilin-1 (PSI) cause familial Alzheimer disease (FAD). The results described herein show that PSI is highly concentrated in "bridges" connecting the endoplasmic reticulum and mitochondria. Mutated PSI increases this communication, resulting in many of the biochemical features that are hallmarks of FAD. Studying this relationship can indicate pathogenesis and therapeutic approaches for this devastating disease.

[00640] Presenilin 1 (PSI) localizes to the plasma membrane (PM), where it contributes to processing and accumulation of extracellular β-amyloid as part of the γ-secretase complex. In addition to this well established function, the results described herein show that PSI plays another role in the pathogenesis of AD. Previous studies have revealed that PSI is targeted not only to the PM, but also to the endoplasmic reticulum (ER). The results described herein show that PSI is enriched in a specific subcompartment of the ER that is associated intimately with mitochondria and that forms a physical bridge between the two organelles, called ER mitochondria-associated membranes (MAM). As described herein, defects in mitochondrial distribution and morphology, as well as alterations in bioenergetics, redox levels, and Ca2+ homeostasis have also exist in various PSI mutant cells.

[00641] ER-MAM has known functions in calcium homeostasis and mitochondrial distribution, two processes that affect synaptic transmission, which is known to be compromised in AD patients. Since defects in the accumulation of mitochondria at the synapse and defects in mitochondrial fusion and fission impair synaptic transmission, mitochondrial distribution and morphology defects can contribute significantly to the pathogenesis of FADPSI. As described herein mutations in PSI inhibit mitochondrial distribution and hence neuronal transmission through effects on mitochondrial-ER interactions. Since Ca2+ regulates the attachment of mitochondria to microtubules, the defects in mitochondrial distribution observed FADPSI cells can be due to defects in ER-MAM-mediated calcium homeostasis that alter axonal mitochondrial transport. Alternatively, since ER-MAM has been shown to contribute to the anchorage of mitochondria at sites of
polarized cell surface growth, the accumulation of mitochondria in the nerve terminal can be compromised in PS1 mutants. These two models are not mutually exclusive. The specific aims described herein are designed to determine the mechanism(s) underlying defects in mitochondrial distribution in PS1 mutants, and to address the role of ER-MAM-targeted PS1 in those processes.

[00642] Without wishing to be bound by theory, mutations in PS1 may inhibit mitochondrial distribution and hence neuronal transmission through effects on mitochondrial-ER interactions, via potential alterations in Ca2+ homeostasis, axonal mitochondrial transport, and/or anchorage of the organelle in the synapse. The maldistribution of mitochondria would be deleterious in elongated neurons, where mitochondria travel vast distances on microtubules to provide ATP for energy-intensive processes at distal sites, including synapses. Mitochondrial distribution and morphology can be studied in neurons from normal and FADPS1 patients and PS1-mutant transgenic mice and (b) the effect of PS1 mutations on mitochondrial dynamics can be analyzed (i.e. transport, retention, fusion, and fission) in these neurons under different excitation states, using mitochondrially-targeted photoactivable fluorescent probes ("mitoDendra") and live-cell imaging. The role of PS1 in ER-MAM can be investigated by (a) studying mitochondrial bioenergetics and redox signaling, using well-established methodologies, (b) analyzing Ca2+ homeostasis in PS1-mutated cells, using Ca2+-sensitive GFPs ("pericams"), (c) examining mitochondrial dynamics, neuronal transmission, and Ca2+ homeostasis after disrupting ER-mitochondrial interactions genetically in PACS2-KO mice, and (d) assessing the role of PS1 in maintaining ER-MAM function. A combination of blue native gels, immunoprecipitation, and protein identification methods will be used to determine whether PS1 has ER-MAM specific protein partners.

[00643] Example 60: Mitochondrial Mislocalization in Disease

These results are supported by (1) the observation that a PS1 mutation (M1 46V) in a mouse PS1 knock-in model impairs axonal transport and also increases tau phosphorylation (Pigino et al. (2003) J. Neurosci. 23:4499-4508), (2) the finding of axonal defects, consisting of swellings that accumulate abnormal amounts of microtubule-associated and molecular motor proteins, organelles, and vesicles, in SAD patients and in transgenic mouse models of AD (Stokin et al. (2005) Science 307:1282-1288), and (3) the identification of a few rare patients with inherited frontotemporal dementia (Pick disease) (Dermaut et al. (2004) Ann. Neurol. 55:617-626; Halliday et al. (2005) Ann. Neurol. 57:139-143) and inherited dilated cardiomyopathy (Li et al. (2006) Am. J. Hum. Genet. 79:1030-1039) who had mutations in PS1 but who did not accumulate Aβ deposits in affected tissues; these "outlier" patients indicate that a clinical presentation due to mutations in PS1 can be "uncoupled" from the morphological hallmarks of AD. PS1 is physically and functionally associated with ER-MAM, and that mutations in PS1 which affect warrants further investigation.

In addition to showing how PS1 functions in ER-mitochondrial communication, the analysis of ER-MAM function can also be used to define a strategy for treating FADPS1. Because altered ER-MAM function is, in all or some aspects, the underlying pathogenetic cause of FAD, approaches to improve this function are therapeutically valuable. Both the SCD1/DGAT2 FRET assay and the cinnamycin toxicity assay can be used in a large-scale chemical screen of PS1-mutant cells to identify compounds that rescue FRET and/or cinnamycin sensitivity in colorimetric assays.

REFERENCES


Andjelic CD, Panelles V, Barrows LR. Characterizing the anti-HIV activity of papuamide A. Mar Drugs 2008;6:528-549.


Borchelt et al, Neuron, 1996, 17, 1005-1013


members of the prohibitin family of proteins that define lipid-raft-like domains of the ER. J Cell Sci 119: 3149-3160


Ganes et. al, 1995, Nature 373:523


Presenilin-1 forms complexes with the cadherin/catenin cell-cell adhesion system and is recruited to intercellular and synaptic contacts. Mol. Cell. 4:893-902.


Guo Q, Sebastian L, Sopher BL, Miller MW, Ware CB, Martin GM, Mattson MP. (1999) Increased vulnerability of hippocampal neurons from presenilin-1 mutant knock-in mice to amyloid β-peptide toxicity: central roles of superoxide production and caspase activation. J. Neurochem., 72, 1019-1029.


Hayashi T, Su TP (2007) Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca(2+) signaling and cell survival. Cell 131:596-610.


Jousset H, Frieden M, Demaurex N (2007) STIM1 knockdown reveals that store-operated Ca2+ channels located close to sarco/endoplasmic Ca2+ ATPases (SERCA) pumps silently refill the endoplasmic reticulum. J. Biol. Chem. 282:1 1456-1 1464.


- 195 -


Shen et al, Cell, 1997, 89, 629-639


Zampese E, Fasolato C, Kipanyula MJ, Bortolozzi M, Pozzan T, Pizzo P (2011) Presenilin 2 - 200 -
modulates endoplasmic reticulum (ER)-mitochondria interactions and Ca2+ cross-talk. Proc Natl Acad Sci USA 108: 2777-2782


WHAT IS CLAIMED IS:

1. A method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising:

   (a) obtaining a test biological sample from a subject,
   
   (b) measuring the rate of conversion of phosphatidylserine to phosphatidylethanolamine in the test biological sample of step (a),
   
   (c) comparing the rate of conversion of phosphatidylserine to phosphatidylethanolamine measured in step (b) to the rate of conversion of phosphatidylserine to phosphatidylethanolamine in a control biological sample, wherein a greater rate of conversion of phosphatidylserine to phosphatidylethanolamine measured in the test biological sample of step (b) relative to the rate of conversion of phosphatidylserine to phosphatidylethanolamine measured in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

2. A method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising:

   (a) obtaining a test biological sample from a subject,
   
   (b) measuring the amount of localization of ER-MAM associated proteins to ER-MAM in the test biological sample of step (a),
   
   (c) comparing the amount of localization of ER-MAM associated proteins to ER-MAM measured in step (b) to the amount of localization of ER-MAM associated proteins to ER-MAM in a control biological sample, wherein an altered amount of localization of ER-MAM associated proteins to ER-MAM measured in the test biological sample of step (b) relative to the amount of localization of ER-MAM
associated proteins to ER-MAM in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

3. A method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising:

   (a) obtaining a test biological sample from a subject,

   (b) measuring the amount of ER-MAM in the test biological sample of step (a),

   (c) comparing the amount of ER-MAM measured in step (b) to the amount of ER-MAM in a control biological sample, wherein an altered amount of ER-MAM measured in the test biological sample of step (b) relative to the amount of ER-MAM in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

4. A method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising:

   (a) obtaining a test biological sample from a subject,

   (b) measuring the activity of one or more non-presenilin ER-MAM-associated proteins in the test biological sample of step (a),

   (c) comparing the activity of one or more non-presenilin ER-MAM-associated proteins measured in step (b) to the activity of one or more non-presenilin ER-MAM-associated proteins in a control biological sample, wherein an altered activity of the one or more non-presenilin ER-MAM-associated proteins measured in the test biological sample of step (b) relative to the activity of the one or more non-presenilin ER-MAM-associated proteins in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

5. A method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising:

   (a) obtaining a test biological sample from a subject,
(b) measuring the amount of functional presenilin in ER-MAM in the test biological sample of step (a),

(c) comparing the amount of functional presenilin in ER-MAM measured in step (b) to the amount of functional presenilin in ER-MAM in a control biological sample, wherein a lesser amount of functional presenilin in ER-MAM measured in the test biological sample of step (b) relative to the amount of functional presenilin in ER-MAM in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

6. A method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising:

(a) obtaining a test biological sample from a subject,

(b) measuring the amount of association between ER-MAM associated proteins in the test biological sample of step (a),

(c) comparing the amount of association between ER-MAM associated proteins measured in step (b) to the amount of association between ER-MAM associated proteins in a control biological sample, wherein an altered amount of association between ER-MAM associated proteins measured in the test biological sample of step (b) relative to the amount of association between ER-MAM associated proteins in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

7. A method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising:

(a) obtaining a test biological sample from a subject,

(b) measuring the sensitivity to cinnamycin induced cell death in the test biological sample of step (a),

(c) comparing the sensitivity to cinnamycin induced cell death measured in step (b) to the sensitivity to cinnamycin induced cell death in a control biological sample, wherein an greater sensitivity to cinnamycin induced cell death measured
in the test biological sample of step (b) relative to the sensitivity to cinnamycin induced cell death in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

8. A method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising:

(a) obtaining a test biological sample from a subject,

(b) measuring the amount of communication between ER and mitochondria in the test biological sample of step (a),

(c) comparing the amount of communication between ER and mitochondria measured in step (b) to the amount of communication between ER and mitochondria in a control biological sample, wherein a greater amount of communication between ER and mitochondria measured in the test biological sample of step (b) relative to the amount of communication between ER and mitochondria in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

9. A method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising:

(a) obtaining a test biological sample from a subject,

(b) measuring the sensitivity to apoptogen induced cell death in the test biological sample of step (a),

(c) comparing the sensitivity to apoptogen induced cell death measured in step (b) to the sensitivity to apoptogen induced cell death in a control biological sample, wherein an greater apoptogen to cinnamycin induced cell death measured in the test biological sample of step (b) relative to the sensitivity to apoptogen induced cell death in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

10. A method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising:
(a) obtaining a test biological sample from a subject,

(b) measuring the amount of ACATI activity in the test biological sample of step (a),

(c) comparing the amount of ACATI activity measured in step (b) to the amount of ACATI activity in a control biological sample, wherein a greater amount of ACATI activity measured in the test biological sample of step (b) relative to the amount of ACATI activity in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

11. A method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising:

   (a) obtaining a test biological sample from a subject,

   (b) measuring the amount of cholesterol ester synthesis in the test biological sample of step (a),

   (c) comparing the amount of cholesterol ester synthesis measured in step (b) to the amount of cholesterol ester synthesis in a control biological sample, wherein a greater amount of cholesterol ester synthesis measured in the test biological sample of step (b) relative to the amount of cholesterol ester synthesis in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

12. A method for determining whether a subject is predisposed to having Alzheimer's disease, the method comprising:

   (a) obtaining a test biological sample from a subject,

   (b) measuring the amount of lipid droplet formation in the test biological sample of step (a),

   (c) comparing the amount of lipid droplet formation measured in step (b) to the amount of lipid droplet formation in a control biological sample, wherein a greater amount of lipid droplet formation measured in the test biological sample of step
(b) relative to the amount of lipid droplet formation in the control biological sample indicates that the subject is predisposed to having Alzheimer's disease.

13. A method for determining whether a compound ameliorates Alzheimer's disease in a subject, the method comprising determining whether a test compound is capable of (a) decreasing the amount of phosphatidylserine conversion to phosphatidylethanolamine in cells contacted with the test compound as compared to cells not contacted with the test compound; (b) increasing amount of functional presenilin in ER-MAM in cells contacted with the test compound as compared to cells not contacted with the test compound; (c) decreasing sensitivity to cinnamycin induced cell death in cells contacted with the test compound as compared to cells not contacted with the test compound; (d) decreasing communication between ER and mitochondria in cells contacted with the test compound as compared to cells not contacted with the test compound; (e) decreasing cell death in response to an apoptogen in cells contacted with the test compound as compared to cells not contacted with the test compound; (f) decreasing ACATI activity in cells contacted with a test compound as compared to cells not contacted with the test compound; (g) decreasing cholesterol ester synthesis in cells contacted with the test compound as compared to cells not contacted with the test compound; (h) decreasing lipid droplet formation in cells contacted with the test compound as compared to cells not contacted with the test compound, or any combination thereof.
Figure 1
Figure 2
Figure 3
Figure 5
Figures 6A - 6C
Figures 8A - 8B
Figures 8C - 8D
Figures 8E - 8F
Figure 9
<table>
<thead>
<tr>
<th>Marker for:</th>
<th>Total</th>
<th>CM</th>
<th>PM</th>
<th>MAM</th>
<th>Mito</th>
<th>ER</th>
<th>Antibody to:</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cadherin (CDH2) (~100 kDa)</td>
</tr>
<tr>
<td>Golgi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GM130 (GOLGA2) (~112 kDa)</td>
</tr>
<tr>
<td>Mito</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Complex I (NDUFA9) (~43 kDa)</td>
</tr>
<tr>
<td>ER</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Calnexin (CANX) (~68 kDa)</td>
</tr>
<tr>
<td>MAM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ACAT1 (~65 kDa)</td>
</tr>
<tr>
<td>MAM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>G6PC (~41 kDa)</td>
</tr>
<tr>
<td>MAM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PEMT (~22 kDa)</td>
</tr>
</tbody>
</table>

**Figure 10**
Figure 11A - 11B
Figure 12A - 12C
Figure 13A – 13B
Figures 15A - 15C
Figure 16
Figure 19
Figure 22
Figure 23
Figure 24A-24B
Figure 25B

Pericam: Mitochondrial [Ca^{2+}]m

CaCl\textsubscript{2}  ATP  Tg  PS1-KD (M2)  Control (M3)

3.5  2.5  2.0  1.5  1.0  0.5  0.0

[Y-axis: time (sec)]

[B-axis: [Ca^{2+}]m, F/F\textsubscript{0}]
## Figure 27

**A**

![Diagram of MAM, ER, and related mitochondrial association](image)

**B**

<table>
<thead>
<tr>
<th>Marker for</th>
<th>Total</th>
<th>Nuc</th>
<th>PM</th>
<th>MAM &quot;MER&quot;</th>
<th>ER</th>
<th>&quot;Free&quot; Mitos</th>
<th>Antibody to</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td><img src="image" alt="Image" /></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Calnexin</td>
</tr>
<tr>
<td>PM</td>
<td><img src="image" alt="Image" /></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cacherin</td>
</tr>
<tr>
<td>Golgi</td>
<td><img src="image" alt="Image" /></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GM130</td>
</tr>
<tr>
<td>Mitochondria</td>
<td><img src="image" alt="Image" /></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NDUFA9</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Image" /></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PS1 - C-term</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Image" /></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Aβ42 (Chemicon AB5078P)</td>
</tr>
</tbody>
</table>
### Figure 28

#### A

<table>
<thead>
<tr>
<th>Marker for</th>
<th>PM</th>
<th>MAM</th>
<th>Mito</th>
<th>ER</th>
<th>Antibody to</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td>Na,K-ATPase (~100 kDa)</td>
</tr>
<tr>
<td>Golgi</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td>GM130 (~112 kDa)</td>
</tr>
<tr>
<td>MAM</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
<td>IP3R3 (~200 kDa)</td>
</tr>
<tr>
<td>Mito</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
<td><img src="image16.png" alt="Image" /></td>
<td>ATP synthase-α (~60 kDa)</td>
</tr>
<tr>
<td>ER</td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
<td><img src="image19.png" alt="Image" /></td>
<td><img src="image20.png" alt="Image" /></td>
<td>SSR-α (~35 kDa)</td>
</tr>
</tbody>
</table>

#### B

<table>
<thead>
<tr>
<th>PM</th>
<th>MAM</th>
<th>Mito</th>
<th>ER</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image21.png" alt="Image" /></td>
<td><img src="image22.png" alt="Image" /></td>
<td><img src="image23.png" alt="Image" /></td>
<td><img src="image24.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="image25.png" alt="Image" /></td>
<td><img src="image26.png" alt="Image" /></td>
<td><img src="image27.png" alt="Image" /></td>
<td><img src="image28.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="image29.png" alt="Image" /></td>
<td><img src="image30.png" alt="Image" /></td>
<td><img src="image31.png" alt="Image" /></td>
<td><img src="image32.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="image33.png" alt="Image" /></td>
<td><img src="image34.png" alt="Image" /></td>
<td><img src="image35.png" alt="Image" /></td>
<td><img src="image36.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="image37.png" alt="Image" /></td>
<td><img src="image38.png" alt="Image" /></td>
<td><img src="image39.png" alt="Image" /></td>
<td><img src="image40.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 29
Figure 30A-30E
Figure 31
Figure 32A-B
Figure 32C-D
Figure 33A-B
Figure 36
Figure 37
Figure 38A-38C
Figure 39A-39F
Figure 39G
Figure 39I
Figure 41A-41D
Figure 41E
Figure 42
Figure 43
Figure 44B
Figure 44C
Figure 44D
Figure 45A
Figure 45B
Figure 46A-46B
Figure 46C-46D
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>Mutation</th>
<th>Age</th>
<th>Sex</th>
<th>LipidTox</th>
<th>Oil</th>
<th>O-JH-Chol</th>
<th>JH-Ser</th>
<th>CIN</th>
<th>FL-SA-Ro</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORH</td>
<td>Fibroblast</td>
<td>Control</td>
<td>36</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LUT</td>
<td>Fibroblast</td>
<td>Control</td>
<td>31</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>FSP</td>
<td>Fibroblast</td>
<td>Control</td>
<td>31</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>AGD7871</td>
<td>Fibroblast</td>
<td>Control</td>
<td>49</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>AGD8379</td>
<td>Fibroblast</td>
<td>Control</td>
<td>60</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>AGD8909</td>
<td>Fibroblast</td>
<td>Control</td>
<td>73</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>AGD8517</td>
<td>Fibroblast</td>
<td>Control</td>
<td>66</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>AGD8525</td>
<td>Fibroblast</td>
<td>Control</td>
<td>82</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>AGD6640</td>
<td>Fibroblast</td>
<td>FAD-P51(A246E)</td>
<td>56</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AGD6048</td>
<td>Fibroblast</td>
<td>FAD-P51(A246E)</td>
<td>56</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AGD3133</td>
<td>Fibroblast</td>
<td>FAD-P51(M146L)</td>
<td>18</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EB</td>
<td>Fibroblast</td>
<td>FAD-P51(G209V)</td>
<td>41</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WA</td>
<td>Fibroblast</td>
<td>FAD-P51(S141F)</td>
<td>33</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DD</td>
<td>Fibroblast</td>
<td>FAD-P52(V141I)</td>
<td>48</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AGD4402</td>
<td>Fibroblast</td>
<td>SAD</td>
<td>47</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AGD5816</td>
<td>Fibroblast</td>
<td>SAD</td>
<td>79</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AGD6283</td>
<td>Fibroblast</td>
<td>SAD</td>
<td>67</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AGD6669</td>
<td>Fibroblast</td>
<td>SAD</td>
<td>60</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AGD1978</td>
<td>Fibroblast</td>
<td>SAD</td>
<td>87</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AGD1366</td>
<td>Fibroblast</td>
<td>SAD</td>
<td>50</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AGD1414</td>
<td>Fibroblast</td>
<td>SAD</td>
<td>39</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AGD1449</td>
<td>Fibroblast</td>
<td>SAD</td>
<td>89</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AGD1158</td>
<td>Fibroblast</td>
<td>SAD</td>
<td>69</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PS-WT</td>
<td>Mouse MEF</td>
<td>Wild-type control</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PS1-KO</td>
<td>Mouse MEF</td>
<td>P51-KO</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ps2-KO</td>
<td>Mouse MEF</td>
<td>P52-KO</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DKO</td>
<td>Mouse MEF</td>
<td>P51+P52 double KO</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M3</td>
<td>Mouse MEF</td>
<td>P51-KD control</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M2x2</td>
<td>Mouse MEF</td>
<td>P51-KD</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M2x2+WT</td>
<td>Mouse MEF</td>
<td>P51-KD + Human WT-P51</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M2x2+246E</td>
<td>Mouse MEF</td>
<td>P51-KD + Human A246E P51</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S10</td>
<td>Mouse MEF</td>
<td>Wild-type control</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S11</td>
<td>Mouse MEF</td>
<td>Mfn2-KO</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S16</td>
<td>Mouse MEF</td>
<td>Mfn2-KO</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S18</td>
<td>Mouse MEF</td>
<td>Wild-type control</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Figure 47**
Figure 48
Figure 49A
AD Fibroblasts

Figure 49B
AD fibroblasts

Control (AG08517)  Control (ESP)

FAD-PS1 (AG06040)  FAD-PS1 (WA)

FAD-PS1 (AG06048)  SAD (AG11414)

FAD-PS2 (DD)  SAD (AG21158)

Figure 50