Supporting Information

Self-Assembling NanoLuc Luciferase Fragments as Probes for Protein Aggregation in Living Cells

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1. Stock Solutions

1x phosphate buffered saline (1x PBS): 137 mM NaCl, 2 mM KH_2PO_4,7.9 mM Na_2HPO4, 2.7 mM KCl, pH = 7.4. 2 mM 2-mercaptoethanol was added to the buffer prior to use.

2x Nluc assay buffer[1]: 100 mM MES pH = 6.0, 1 mM EDTA, 150 mM KCl, 2 mM 2-mercaptoethanol, and 35 mM thiourea. This stock buffer was diluted with nanopure water to obtain 1x Nluc assay buffer.

Coelenterazine working stock: Coelenterazine was dissolved in acidified ethanol and diluted to 50 µM using 1x Nluc assay buffer.

Ampicillin stock solution: 1 g ampicillin was dissolved into 10 mL of nanopure water to yield 100 mg/ml stock solution (1000x). The solution was filtered through a 0.2 µm sterile syringe filter, aliquoted, and stored at -20 ^{o}C.

Kanamycin stock solution: 0.5 g kanamycin was combined with 10 mL of nanopure water to yield a 50 mg/ml stock solution (1000x). The resulting solution was filtered through a 0.2 µm sterile syringe filter, aliquoted, and stored at -20 ^{o}C.

TBST solution (10x): 200 mM Tris-HCl pH = 7.5, 1.5 M NaCl, and 1% Tween-20. Diluted to 1x TBST using nanopure water.

2. Identification of Potential Nluc Fragmentation Sites

In order to identify potential fragmentation sites in Nluc, we obtained Kyte values[2] for the Nluc amino acid sequence. In addition, the secondary structure of Nluc was predicted using PSIPRED.[3] Combining the information obtained from both these analyses, we selected 5 sites located at predicted hydrophilic loop regions and studied the activity of the corresponding fragmented pairs. The amino acid residue numbering of these fragmentation sites is given below.

	Nluc Fragmentation Sites	
	N-Terminal Fragment[a]	C-Terminal Fragment[a]
N65/66C	1-65	66-171
N76/77C	1-76	77-171
N87/88C	1-87	88-171
N116/117C	1-116	117-171
N137/138C	1-137	138-171

[a]Numbering indicates amino acid residue.

3. Cloning of Nluc Fragments

DNA sequences encoding N-terminal fragments of Nluc[1] were amplified using primers containing 5' SacI and 3' HindIII restriction sites for incorporation into the pET-45b vector (Novagen). Similarly, DNA sequences corresponding to C-terminal Nluc fragments were amplified and incorporated into the pRSF-1b vector (Novagen) using NcoI and PstI restriction sites. Primers also encoded an amino-terminal hexahistidine tag for the N-terminal fragments. PCR amplified N-terminal Nluc fragments were digested with SacI and HindIII, and ligated to SacI/HindIII digested and calf intestinal phosphatase (CIP)-treated pET-45b. The ligation

mixture was transformed into XL1-Blue competent cells (Stratagene) and grown on ampicillin plates. Plasmid DNA was isolated from selected colonies and confirmed by DNA sequencing. In the same fashion, the PCR amplified C-terminal Nluc fragments were digested with NcoI and PstI, and ligated to NcoI/PstI digested and CIP-treated pRSF-1b. The ligation mixture was transformed into XL1-Blue competent cells, grown on kanamycin plates, and positive clones were confirmed by DNA sequencing.

4. Cloning of Amyloid-Beta-, GFP-, and MBP-N65 Fusions

DNA sequences encoding the maltose binding protein (MBP) were amplified using primers containing 5' BamHI and 3' XmaI restriction sites for incorporation into the pETNluc N65 vector. Amyloid-beta and GFP sequences were incorporated into the pETNluc N65 vector via 5' BamHI and 3' BglII restriction sites. The resulting N-terminal N65 fusion proteins had an N-terminal hexahistidine tag and a GGGSSGGG linker between the fusion protein and N65. Amyloid-beta mutants[4] were generated using site-directed mutagenesis (QuikChange Lightning Multi Site-Directed Mutagenesis Kit, Agilent) using wild-type amyloid-beta as a template, according to manufacturer protocols.

5. Preparation of Extracts Containing Total Protein or Soluble Protein Fractions of Amyloid-Beta Mutants

BL21-Gold (DE3) cells were cotransformed with vectors containing N-terminal fusions of wild-type amyloid-beta or the indicated amyloid-beta mutants fused to N65 along with a vector expressing 66C. Single colonies were used to inoculate 6 mL of TB media containing ampicillin and kanamycin. Cultures were grow to an OD_{600} = 0.6 – 0.8 at 37 °C, with shaking at 250 rpm. Cultures were then cooled to 16 °C, and the cells were induced with 0.2 mM IPTG and grown overnight. The next morning, an amount of cells equal to an OD_{600} = 3.0 in 1 mL was harvested by centrifugation at 17,000g for 10 min and resuspended in 200 µL of 8 M urea and 200 µl of 10% SDS. The cell samples were heated at 100 °C for 10 minutes, centrifuged for 15 minutes at 17,000g, and lysates were incubated with 10 µL HisPur Ni-NTA resin at 4 °C with rotation for an hour. The resin was collected by centrifugation at 500g for 10 min and washed with 200 µL of 1x PBS containing 10 mM imidazole and 8 M urea. Proteins were eluted by adding 10 µL of 1x PBS containing 1 M imidazole and 8 M urea followed by immediate addition of 10 µl of SDS-PAGE loading dye solution. After heating at 100 °C for 10 minutes, the samples were centrifuged for 15 minutes at 17,000g. The resulting supernatants (2.5 µL), which represent the total expression levels of N65 fusions and 66C, were loaded onto a 12% polyacrylamide gel and analyzed by Western blotting.

For isolation of proteins in the soluble fraction, cells were induced using the protocol described above and normalized to an OD_{600} = 3.0 in 1 mL. Cells were lysed, according to the vender provided protocol, with 200 µl of B-PER reagent. The resulting soluble fractions were incubated with 10 µl of HisPur Ni-NTA resin, as described above except that urea was not included during the washing or elution steps. Soluble proteins (10 µL) were loaded onto a 12% polyacrylamide gel and analyzed by Western blotting.

6. Western Blotting Conditions.

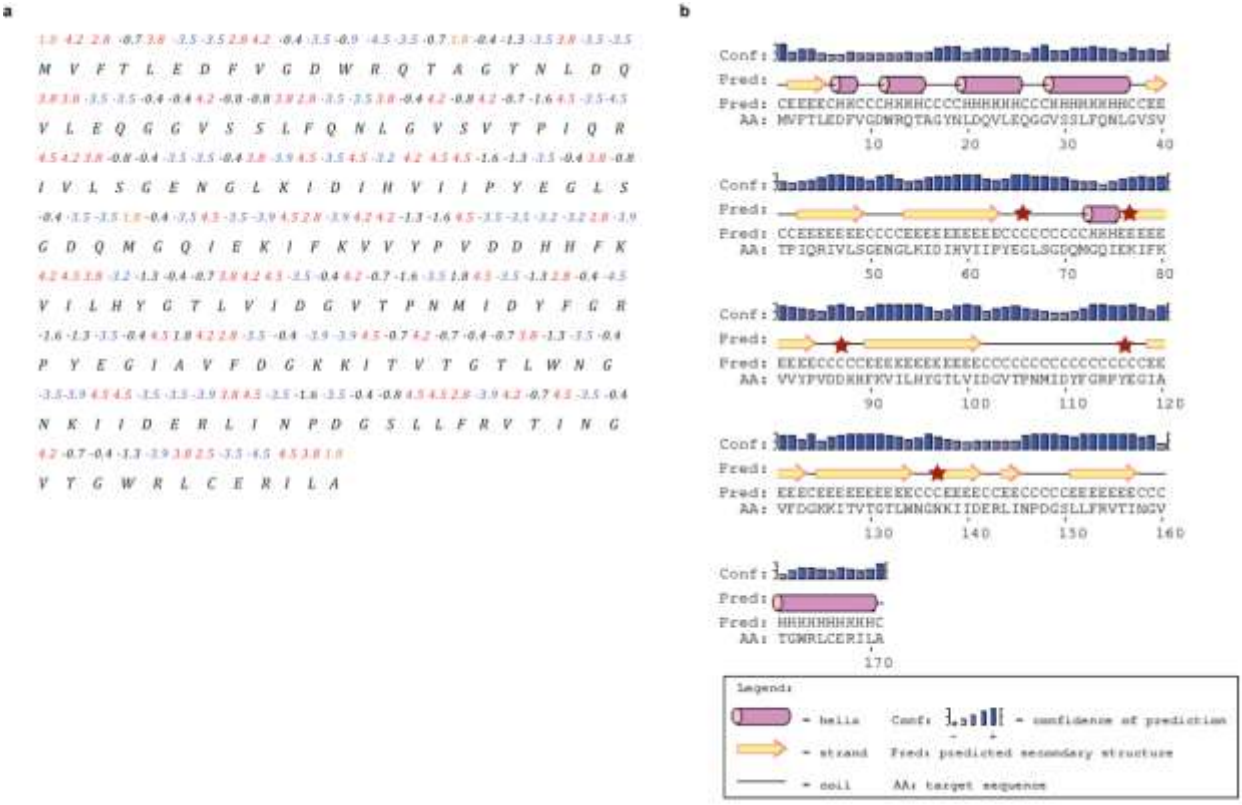
Cell samples were normalized to an OD_{600} = 3.0 in 1 mL. Cells were harvested by centrifugation at 17,000g for 10 min at 4 °C and were lysed using the B-PER Bacterial Protein Extraction Reagent (100 µL). Lysates were clarified by centrifugation and incubated with 10 µL Ni-NTA resin for 1 hr at 4 °C with rotation. Ni-NTA resin was then collected via centrifugation at 800g for 5 min and washed with increasing concentrations of imidazole in 1x PBS (100 µL of 10 mM imidazole followed by 100 µL of 50 mM imidazole). Resin was resuspended in 5 µL 500 mM imidazole and 5 µL SDS-PAGE loading dye. After denaturing at 95 °C for 10 minutes, the samples were centrifuged at 17,000g for 5 min and cooled to room temperature before loading on a 12% SDS-PAGE gel. Samples were transferred from the SDS-PAGE gel to a nitrocellulose membrane using standard protocols. The membrane was blocked with 5% milk in 1x TBST for 1 hr at room temperature. The membrane was washed three times with 1x TBST (15 minutes per wash), before incubating with a 1:10,000 dilution of anti-6X His-tag antibody conjugated to HRP (Abcam, ab1187) in 1x TBST containing 5% milk overnight at 4 °C with rotation. The next morning, the membrane was washed three times with 1x TBST (1 hr for the first wash, and 15 min for the second and third wash). Bands were visualized using the SuperSignal West Dura Extended Duration Substrate (Life Technologies, 34075) on a Gel Doc XR+ system.

7. Cloning of Mammalian Cell Expression Constructs.

DNA sequences encoding N-terminal fragments of Nluc were amplified and ligated into the mVenus C1 vector (Addgene, 27794). Similarly, DNA sequences corresponding to C-terminal Nluc fragments were amplified and ligated into the mCerulean N1 vector (Addgene, 27795). Fusion proteins were amplified and ligated into the mVenus C1 vector containing the N65 Nluc fragment.

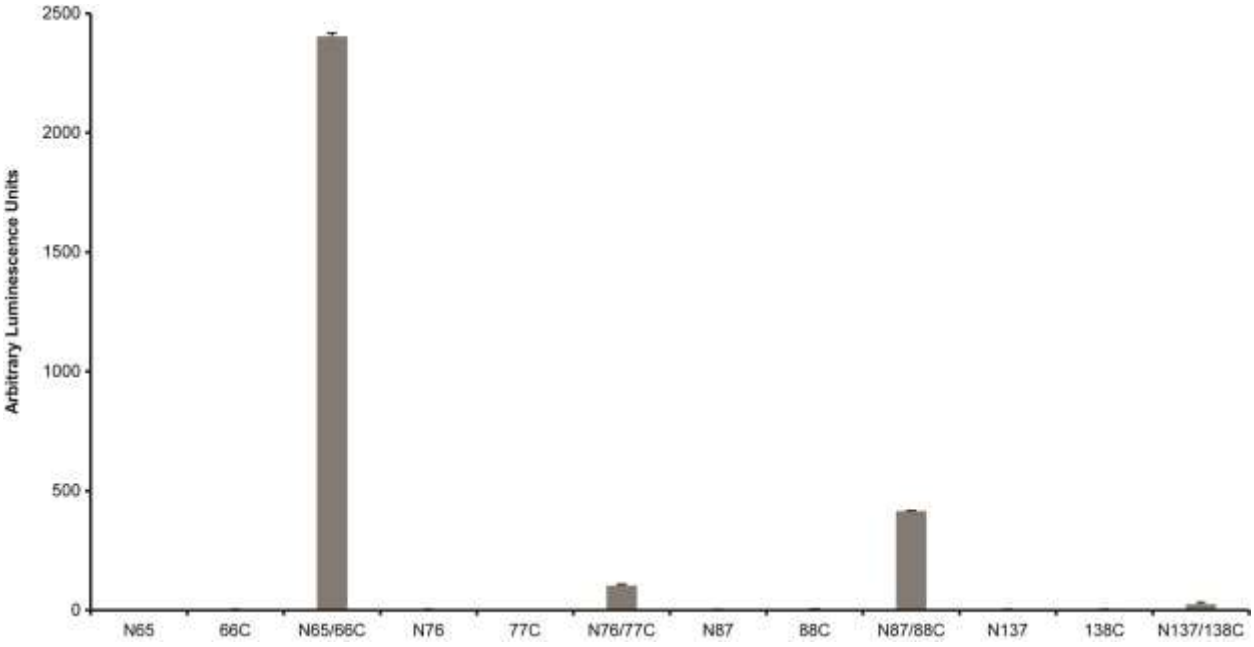
8. Supplementary Figures

Figure S1



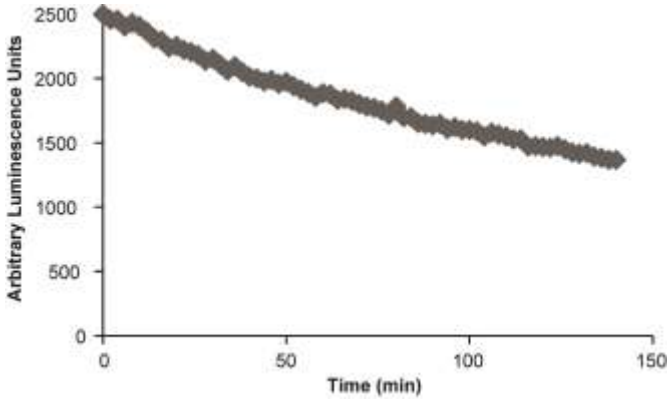
Identification of potential Nluc fragmentation sites. (a) Kyte values[2] for the Nluc amino acid sequence. Red values indicate hydrophobic residues while blue values indicate hydrophilic residues. (b) The predicted secondary structure of Nluc obtained using PSIPRED.[3] Red stars indicate sites chosen for fragmentation.

Figure S2



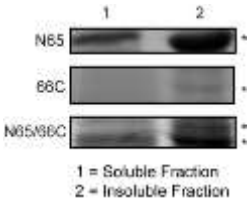
Luminescence intensity from bacterial lysates containing the indicated Nluc fragment. Individual lysates are mixed where indicated. Lysates were normalized for cell density and error bars represent the standard deviation of triplicate experiments.

Figure S3



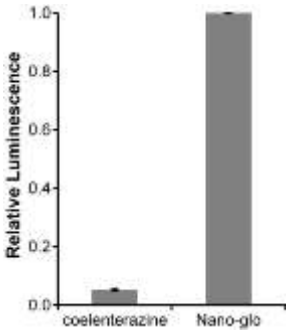
Luminescence signal of the N65/66C lysate mixture from Figure S2 monitored over time.

Figure S4



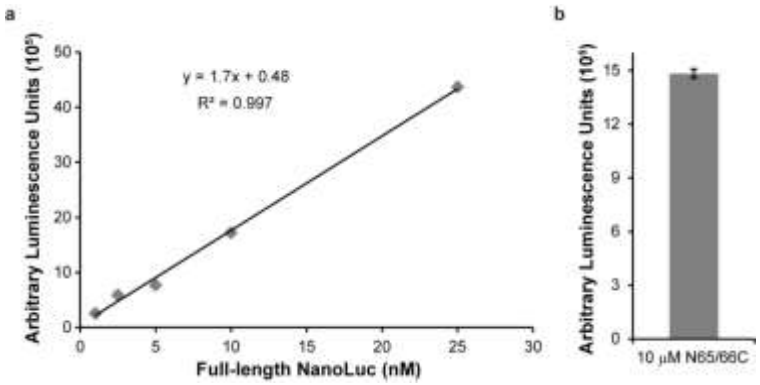
Coomassie stained gels of soluble and insoluble protein fractions from N65 (10.3 kDa) and 66C (13.5 kDa) expressed alone or together. Coexpression leads to an increase in the total expression of 66C. Bands corresponding to the molecular weight of N65 and 66C are indicated by an asterisk.

Figure S5



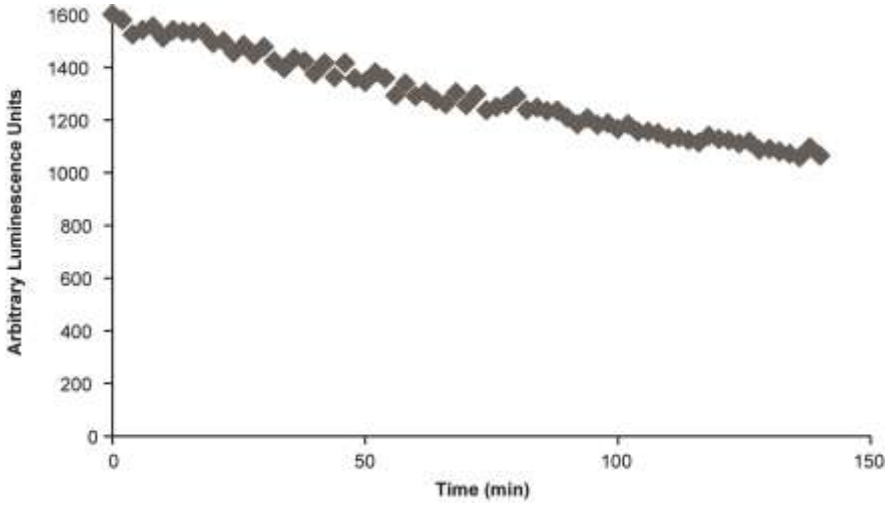
Reassembled Nluc (500 nM each fragment) can utilize either coelenterazine (25 μM) or furimazine as a substrate. Furimazine was added using the Nano-glo substrate (0.25 μL) from Promega. Comparisons of the intensities between coelenterazine and Nano-glo should not be made, as the concentration of furimazine in the commercially available Nano-glo formulation is unknown.

Figure S6



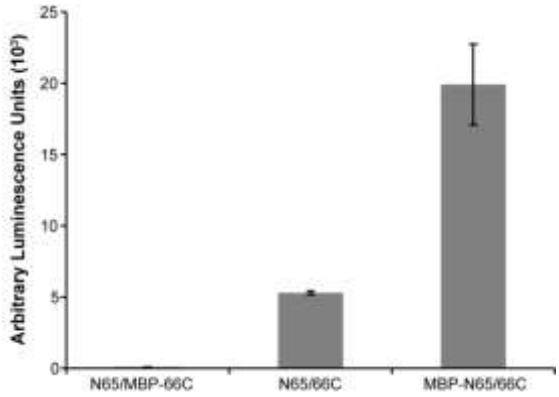
Reassembly efficiency compared to full-length NanoLuc. (a) Luminescence obtained from full-length NanoLuc at varying concentrations in the presence of coelenterazine (25 μM). (b) The luminescence intensity of reassembled N65 (10 μM) and 66C (10 μM) is shown for comparison.

Figure S7



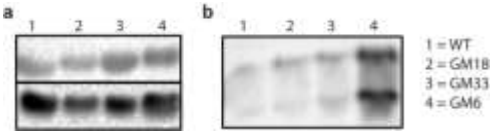
Luminescence signal of a mixture of purified N65 (10 μM) and 66C (50 nM) over time.

Figure S8



Bacterial luminescence of an N-terminal fusion of MBP to 66C (MBP-66C). Dramatically reduced luminescence is observed compared to an N-terminal fusion of MBP to N65 (MBP-N65).

Figure S9



(a) Total expression of N65 fusions (top) and 66C (bottom) does not change based on the identity of the Aβ mutant. (b) The amount of soluble N65 fusion (top) and 66C (bottom) changes according to the known aggregation potential of the appended Aβ mutant.[4] The changes in the relative amount of soluble protein correlate to the luminescence intensities observed in Figure 5a. Procedures for the preparation of samples containing total as well as soluble Aβ mutants are given in the Supporting Information.

9. Supplementary Tables

Table S1: Amino Acid Sequences for Nluc Fragment Constructs

Protein	Amino acid sequence
N65	MAHHHHHHVGTGSNDDDDKSPDPNWELMVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSG ENGLKIDIHVIIPYE
N76	MAHHHHHHVGTGSNDDDDKSPDPNWELMVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSG ENGLKIDIHVIIPYEGLSGDQMGQIE
N87	MAHHHHHHVGTGSNDDDDKSPDPNWELMVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSG ENGLKIDIHVIIPYEGLSGDQMGQIEKIFKVVYPVDD
N116	MAHHHHHHVGTGSNDDDDKSPDPNWELMVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSG ENGLKIDIHVIIPYEGLSGDQMGQIEKIFKVVYPVDDHHFKVILHYGTLVIDGVTPNMIDYFGRPY
N137	MAHHHHHHVGTGSNDDDDKSPDPNWELMVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSG ENGLKIDIHVIIPYEGLSGDQMGQIEKIFKVVYPVDDHHFKVILHYGTLVIDGVTPNMIDYFGRPYEGIAVFDGKKITVTGTL WNGN
66C	MGGLSGDQMGQIEKIFKVVYPVDDHHFKVILHYGTLVIDGVTPNMIDYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLI NPDGSLLFRVTINGVTGWRLCERILALQGSELHHHHHH
77C	MGKIFKVVYPVDDHHFKVILHYGTLVIDGVTPNMIDYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLINPDGSLLFRVTI NGVTGWRLCERILALQGSELHHHHHH
88C	MGHHFKVILHYGTLVIDGVTPNMIDYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLINPDGSLLFRVTINGVTGWRLCE RILALQGSELHHHHHH
117C	MGEGIAVFDGKKITVTGTLWNGNKIIDERLINPDGSLLFRVTINGVTGWRLCERILALQGSELHHHHHH
138C	MGKIIDERLINPDGSLLFRVTINGVTGWRLCERILALQGSELHHHHHH

Table S2: Amino Acid Sequences for N65 Fusion Constructs Used in Bacterial Experiments

Protein	Amino acid sequence
GFP-N65	MAHHHHHHVGTGSNDDDDKSPDPMASMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLT LKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFE GDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIG DGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYKEISYASRGGGSSGGGELMVFTLE DFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIHVIIPYE
MBP-N65	MAHHHHHHVGTGSNDDDDKSPDPMKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKF PQVAATGDGPDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDL LPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAGLT FLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGI NAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEELVKDPRIAATMENAQKGEIMPNIPQMSAF WYAVRTAVINAASGRQTVDEALKDAQTNARGGGSSGGGELMVFTLEDFVGDWRQTAGYNLDQVLEQGGV SSLFQNLGVSVTPIQRIVLSGENGLKIDIHVIIPYE
Aβ$_{1-42}$-N65	MAHHHHHHVGTGSNDDDDKSPDPDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIAEISYAS RGGGSSGGGELMVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIHVII PYE
Aβ$_{1-42}$GM18-N65[a]	MAHHHHHHVGTGSNDDDDKSPDPDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGPMVGGVVIAEISYAS RGGGSSGGGELMVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIHVII PYE
Aβ$_{1-42}$GM33-N65[a]	MAHHHHHHVGTGSNDDDDKSPDPDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGPMVGGVVISEISYAS RGGGSSGGGELMVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIHVII PYE
Aβ$_{1-42}$GM6-N65[a]	MAHHHHHHVGTGSNDDDDKSPDPDAEFRHDSGYEVHHQKLVSFAEDVGSNKGAIIGPMVGGVVIAEISYAS RGGGSSGGGELMVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIHVII PYE
Aβ$_{1-42}$D23N-N65	MAHHHHHHVGTGSNDDDDKSPDPDAEFRHDSGYEVHHQKLVFFAENVGSNKGAIIGLMVGGVVIAEISYAS RGGGSSGGGELMVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIHVII PYE
Aβ$_{1-42}$E22G-N65	MAHHHHHHVGTGSNDDDDKSPDPDAEFRHDSGYEVHHQKLVFFAGDVGSNKGAIIGLMVGGVVIAEISYAS RGGGSSGGGELMVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIHVII PYE

[a]Point mutations are highlighted in red.

Table S3: Amino Acid Sequences for Fusion Proteins Used in Mammalian Cell Experiments

Protein	Amino acid sequence
N65[a]	MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKLICTTGKLPVPWPTLVTTLGYGLQCF ARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEY NYNSHNVYITADKQKNGIKANFKIRHNIEDGGVQLADHYQQNTPIGDGPVLLPDNHYLSYQSKLSKDPNEKR DHMVLLEFVTAAGITLGMDELYKSGLRSAVDGTAGPGSAYASRGGGSSGGGELMVFTLEDFVGDWRQTAG YNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIHVIIPYE
Aβ$_{1-42}$-N65[a]	MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKLICTTGKLPVPWPTLVTTLGYGLQCF ARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEY NYNSHNVYITADKQKNGIKANFKIRHNIEDGGVQLADHYQQNTPIGDGPVLLPDNHYLSYQSKLSKDPNEKR DHMVLLEFVTAAGITLGMDELYKSGLRSRAQAWDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGG VVIAEISYASRGGGSSGGGELMVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGE NGLKIDIHVIIPYE
Aβ$_{1-42}$GM18-N65[a,b]	MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKLICTTGKLPVPWPTLVTTLGYGLQCF ARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEY NYNSHNVYITADKQKNGIKANFKIRHNIEDGGVQLADHYQQNTPIGDGPVLLPDNHYLSYQSKLSKDPNEKR DHMVLLEFVTAAGITLGMDELYKSGLRSRAQAWDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGPMVGG VVIAEISYASRGGGSSGGGELMVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGE NGLKIDIHVIIPYE
Aβ$_{1-42}$GM6-N65[a,b]	MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKLICTTGKLPVPWPTLVTTLGYGLQCF ARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEY NYNSHNVYITADKQKNGIKANFKIRHNIEDGGVQLADHYQQNTPIGDGPVLLPDNHYLSYQSKLSKDPNEKR DHMVLLEFVTAAGITLGMDELYKSGLRSRAQAWDAEFRHDSGYEVHHQKLVSFAEDVGSNKGAIIGPMVGG VVIAEISYASRGGGSSGGGELMVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGE NGLKIDIHVIIPYE
66C[a]	MGGLSGDQMGQIEKIFKVVYPVDDHHFKVILHYGTLVIDGVTPNMIDYFGRPYEGIAVFDGKKITVTGTLWNG NKIIDERLINPDGSLLFRVTINGVTGWRLCERILALQGGGSSGGGKLRILQSTVPRARDPPVATMVSKGEELFT GVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTWGVQCFARYPDHMKQ HDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNAISDNVYITA DKQKNGIKANFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSKLSKDPNEKRDHMVLLEFVTA AGITLGMDELYK

[a]An added fluorescent protein tag is highlighted in blue. [b]Point mutations are highlighted in red.

10. References

(1) Hall, M. P., Unch, J., Binkowski, B. F., Valley, M. P., Butler, B. L., Wood, M. G., Otto, P., Zimmerman, K., Vidugiris, G., Machleidt, T., Robers, M. B., Benink, H. A., Eggers, C. T., Slater, M. R., Meisenheimer, P. L., Klaubert, D. H., Fan, F., Encell, L. P., and Wood, K. V. (2012) Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. *ACS Chem. Biol. 7*, 1848-1857.

(2) Kyte, J., and Doolittle, R. F. (1982) A Simple Method for Displaying the Hydropathic Character of a Protein. *J. Mol. Biol. 157*, 105-132.

(3) Jones, D. T. (1999) Protein secondary structure prediction based on position-specific scoring matrices. *J. Mol. Biol. 292*, 195-202.

(4) Wurth, C., Guimard, N. K., and Hecht, M. H. (2002) Mutations that reduce aggregation of the Alzheimer's Abeta42 peptide: an unbiased search for the sequence determinants of Abeta amyloidogenesis. *J. Mol. Biol. 319*, 1279-1290.