Electronic Supplementary Material (ESI) for Molecular BioSystems.

Supporting Information

Luminescent platforms for monitoring changes in the solubility of amylin and huntingtin in living cells

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1. General Instrumentation and Methods

Luminescence intensities were measured in Corning 96-well assay plates (3359) [white, half-area, round bottom, 120 µL reaction volume] using a SynergyH1 hybrid reader (BioTek). Unless otherwise indicated, assays were conducted in triplicate.

2. Stock Solutions

1x phosphate buffered saline (1x PBS): 137 mM NaCl, 2 mM KH_2PO_4,7.9 mM Na_2HPO_4, 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 and 35 mM thiourea.

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

Ampicillin stock solution: 1g 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 into eppendorf tubes, 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 into eppendorf tubes, 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.

3. Cloning

DNA sequences encoding wild-type amylin were synthesized via overlapping primers (sequences given below), and cloned into the pETNluc N65 vector via BamHI and BglII sites.[2] Amylin mutants I26P and S20G were generated using site-directed mutagenesis (QuikChange Lightning, Agilent) using wild-type amylin as a template, according to manufacturer protocols. Constructs were verified by DNA sequencing.

For constructing Htt fusions to N65, the N65 sequence was amplified and cloned into pBAD_GFP11_T7LysH17A[3] between the XhoI and HindIII sites to generate pBAD_N65_T7LysH17A. DNA sequences encoding Huntingtin exon1 with various polyQ expansions were amplified from pET32a-HD25Q (Addegene, 11508), pET32a-HD46Q (Addegene, 11515), p416 72Q GPD (Addgene, 1179) and p416 103Q GPD (Addgene, 1180).[4,5] The amplified DNA fragments were cloned into pBAD_N65_T7LysH17A via XhoI and SpeI sites. All clones were verified by DNA sequencing.

Primers used for synthesizing wild-type amylin are given below:

Primer name[a]	Primer sequence
AmylinfwdA	CGCGGATCCGAAATGCAACACCGCGACCTGCGCGACCCAGCGCCTGGCGAACTTTC
AmylinfwdB	GCGCCTGGCGAACTTTCTGGTGCATAGCAGCAACAACTTTGGCGCGATTCTGAGCAGC
AmylinrevC	GCGCGATTCTGAGCAGCACCAACGTGGGCAGCAACACCTATCAGATCTCGC

[a]Overlapping sequences are highlighted in blue.

4. Western Blotting Conditions

Extracts of soluble proteins were obtained using the following procedure. Cell samples were normalized to an OD_{600} = 3.0 in 1 mL. Cells were harvested by centrifugation at 700 g for 30 minutes at 4 °C and lysed using the B-PER Bacterial Protein Extraction Reagent (30 µL, Thermo Fisher). The resulting supernatant (8 µL) was mixed was 3µL SDS-PAGE loading dye. After denaturing at 100 °C for 10 minutes, the samples were centrifuged at 17,000g for 5 minutes 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 (5 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 (5 minutes per wash). Bands were visualized using the SuperSignal West Dura Extended Duration Substrate (Life Technologies, 34075) on a Gel Doc XR+ system.

Total protein expression levels were interrogated using the following protocol. Cell samples were normalized to an OD_{600} = 3.0 in 1 mL and were resuspended in 4 M urea containing 5% SDS (60 µL), denatured at 100 °C for 10 minutes, and centrifuged at 17,000 g for 10 minutes. The resulting supernatant (8 µL) was mixed was 3 µL SDS-PAGE loading dye. After denaturing at 100 °C for 10 minutes, the samples were centrifuged at 17,000g for 5 minutes and cooled to room temperature before loading on a 12% SDS-PAGE gel. Western blotting and imaging was performed as described for soluble protein fractions.

5. Amylin Mutant Assays

E. coli BL21-Gold (DE3) cells were freshly cotransformed with pET vectors containing the N-terminal fusions of amylin mutants I26P, S20G, or wild-type amylin to N65 as well as a vector expressing 66C.[2] Single colonies were used to inoculate 8 mL of TB media containing ampicillin and kanamycin. Cultures were grown to an OD_{600} = 0.6-0.8 at 37 °C, 250 rpm. Cells were induced with 1 mM IPTG and grown for 3 hours at 37 °C or were cooled to 16 °C, and induced with 0.2 mM IPTG and grown overnight. Prior to the assay, cell cultures were normalized to an OD_{600} = 3.0 in 1 mL. Cells were harvested by centrifugation at 700 g for 10 minutes at 4 °C and resuspended in 1x Nluc assay buffer (200 µL). An equal volume of cells (195 µL) and 50 µM coelenterazine were mixed in order to initiate the luminescence assay.

6. Amylin Inhibitor Assays

E. coli BL21-Gold (DE3) cells were freshly cotransformed with pET vectors containing the N-terminal fusions of wild-type amylin to N65 as well as a vector expressing 66C.[2] Single colonies were used to inoculate 8 mL of TB media containing ampicillin and kanamycin. Cultures were grow to an OD_{600} = 0.6-0.8 at 37 °C, 250 rpm. Silibinin (5 μM) was added were indicated and cultures were induced with 0.2 mM IPTG and grown for 16 hours, at 16 °C. Prior to the luminescence assay, cell cultures were normalized to an OD_{600} = 3.0 in 1 mL. Cells were harvested by centrifugation at 700 g for 10 minutes at 4 °C and resuspended in 1x Nluc assay buffer (200 μL). An equal volume of cells (195 μL) and 50 μM coelenterazine were mixed in order to initiate the luminescence assay.

7. Htt Assays

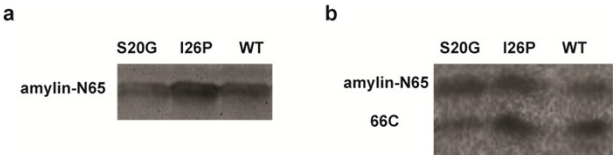
E. coli BL21-Gold (DE3) cells were freshly cotransformed with pBAD vectors containing the N-terminal fusions of Huntingtin mutants Htt46Q, Htt72Q, Htt97Q, or Htt25Q to N65 as well as a vector expressing 66C.[2] Single colonies were used to inoculate 8 mL of TB media containing ampicillin and kanamycin. Cultures were grown to an OD_{600} = 0.6-0.8 at 37 °C, 250 rpm. Cells were induced with 1 mM IPTG and 1% arabinose and grown for 3 hours at 37 °C or were cooled to 16 °C, and induced with 0.2 mM IPTG and 1% arabinose and grown overnight. Prior to luminescence readings, cell cultures were normalized to an OD_{600} = 3.0 in 1 mL. Cells were then harvested by centrifugation at 700g for 10 minutes at 4 °C and resuspended in 1x Nluc assay buffer (200 μL). An equal volume of cells (195 μL) and 50 μM coelenterazine were mixed in order to initiate the luminescence assay.

8. Htt Inhibitor Assays

E. coli BL21-Gold (DE3) cells were freshly cotransformed with a pBAD vector containing an N-terminal fusion of Htt46Q to N65 as well as a vector expressing 66C.[2] Single colonies were used to inoculate 8 mL of TB media containing ampicillin and kanamycin. Cultures were grow to an OD_{600} = 0.6-0.8 at 37 °C, 250 rpm. Cystamine (50 μM) was added were indicated and cultures were induced with 1% arabinose and 0.1 mM IPTG and grown for 16 hours at 16 °C. Cell cultures were normalized to an OD_{600} = 3.0 in 1 mL, harvested by centrifugation at 700g for 10 minutes at 4 °C, and resuspended in 1x Nluc assay buffer (200 μL). An equal volume of cells (195 μL) and 50 μM coelenterazine were mixed in order to initiate the luminescence assay.

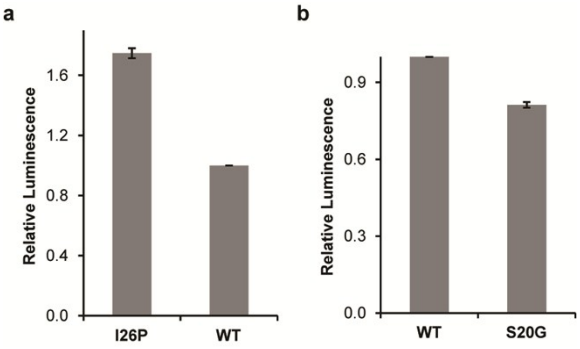
9. Supplementary Figures

Figure S1



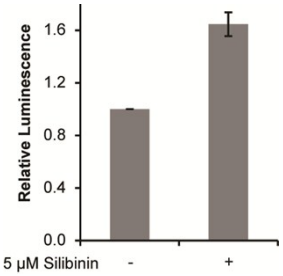
(a) Western blot analysis indicates an increase in soluble protein for the I26P mutant along with a decrease in soluble protein for the S20G mutant, relative to wild-type amylin (WT). (b) These changes in soluble protein levels are not due to changes in total protein expression.

Figure S2



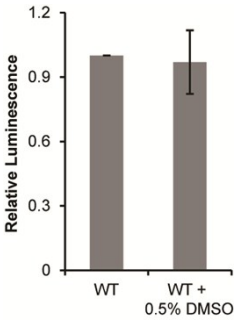
Luminescence intensity of bacterial cells expressing fusions of I26P (a) or S20G (b) mutants to N65, relative to wild-type amylin-N65 (WT). Cells were induced with 1 mM IPTG, at 37 ^{o}C for 3 hrs prior to analysis as in Fig. 2. Experiments were conducted in duplicate.

Figure S3



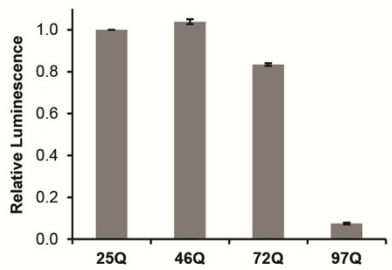
Cells expressing S20G-N65 were incubated without or with 5 µM silibinin. A 60% increase in luminescence signal was observed when cells were treated with the inhibitor. Experiments were conducted in duplicate.

Figure S4



Inhibitor solvent does not influence luminescence. Assays were conducted as in Fig. 3. Luminescence of bacterial cells expressing wild-type amylin-N65 (WT) in absence or presence of 0.5% DMSO (the same concentration DMSO as in Fig. 3). No difference in cellular luminescence is observed.

Figure S5



Luminescence intensity of bacterial cells expressing the indicated Htt fusions to N65. Decreasing luminescence is observed with increasing polyQ length. Cells were induced at 37 ^{o}C for 3 hrs prior to analysis as in Fig. 4. Experiments were conducted in duplicate.

10. Supplementary Tables

Table S1: Amino Acid Sequences for Amylin Fusions to N65

Protein	Amino acid sequence
Amylin-N65	MAHHHHHHVGTGSNDDDDKSPDPKCNTATCATQRLANFLVHSSNN FGAILSSTNVGSNTYQISYASRGGGSSGGGELMVFTLEDFVGDWRQ TAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIHVIIP YE
I26P-N65[a]	MAHHHHHHVGTGSNDDDDKSPDPKCNTATCATQRLANFLVHSSNN FGAPLSSTNVGSNTYQISYASRGGGSSGGGELMVFTLEDFVGDWR QTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIHVII PYE
S20G-N65[a]	MAHHHHHHVGTGSNDDDDKSPDPKCNTATCATQRLANFLVHSGNN FGAILSSTNVGSNTYQISYASRGGGSSGGGELMVFTLEDFVGDWRQ TAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIHVIIP YE

[a]Mutations in amylin are highlighted in red.

Table S2: Amino Acid Sequences for Htt Fusions to N65

Protein	Amino acid sequence
Htt25Q-N65	MGGSHHHHHHGGSTSMATLEKLMKAFESLKSFQQQQQQQQQQQ QQQQQQQQQQQQQQPPPPPPPPPPPQLPQPPPQAQPLLPQPQPP PPPPPPPPGPAVAEEPLHRPLEGGGSSGGGMVFTLEDFVGDWRQT AGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIHVIIPY E
Htt46Q-N65	MGGSHHHHHHGGSTSMATLEKLMKAFESLKSFQQQQQQQQQQQ QQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQPPPPPP PPPPPQLPQPPPQAQPLLPQPQPPPPPPPPPPPGPAVAEEPLHRPLE GGGSSGGGMVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNL GVSVTPIQRIVLSGENGLKIDIHVIIPYE
Htt72Q-N65	MGGSHHHHHHGGSTSMATLEKLMKAFESLKSFQQQQQQQQQQQ QQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQ QQQQQQQQQQQQQQQQQQQQQPPPPPPPPPPPQLPQPPPQAQ PLLPQPQPPPPPPPPPPGPAVAEEPLHRPLEGGGSSGGGMVFTLE DFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGEN GLKIDIHVIIPYE
Htt97Q-N65	MGGSHHHHHHGGSTSMATLEKLMKAFESLKSFQQQQQQQQQQQ QQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQ QQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQ QQQQQQPPPPPPPPPPPQLPQPPPQAQPLLPQPQPPPPPPPPPPG PAVAEEPLHRPLEGGGSSGGGMVFTLEDFVGDWRQTAGYNLDQVL EQGGVSSLFQNLGVSVTPIQRIVLSGENGLKIDIHVIIPYE

11. References

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