Supplementary Information

Laboratory Evolution of a Sortase Enzyme that Modifies the Alzheimer’s Disease-Associated Amyloid β-protein

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Supplementary Table 1. Evolutionary history of SrtAβ. Library size at the beginning of each round, number of sorts before re-diversification, and information on screening stringency are provided. Changes in substrate concentrations and reaction times over the course of a round are indicated where applicable. The sequences in each substrate relevant to sortase recognition are in bold. Changes in incubation time over the course of a round is indicated by an arrow where applicable. TBS-BC = 100 mM Tris pH 7.5, 500 mM NaCl, 1% BSA, 5 mM CaCl$_2$. PC = human plasma, 5 mM CaCl$_2$.

| Round | Library Size | # Sorts | Positive Substrate | Conc. (nM) | Negative Substrate | Conc. (μM) | Time (min) | Buffer |
|-------|--------------|---------|--------------------|------------|-------------------|-------------|------------|--------|
| 1     | 4.8 x 10$^7$ | 5       | Btn-GLPVGGV        | 3200 ➔ 100 | GLPESGT           | 0 ➔ 10     | 60         | TBS-BC |
| 2     | 7.2 x 10$^7$ | 5       | Btn-GLMVGGV        | 10000 ➔ 1000 | LMVTGV LPVGGV | 0 ➔ 100 0 ➔ 100 | 60         | TBS-BC |
| 3     | 3.5 x 10$^7$ | 4       | Btn-GLMVGGV        | 1000 ➔ 320 | LMVTGV LPVGGV | 1 ➔ 20 1 ➔ 20 | 60         | TBS-BC |
| 4     | 1.4 x 10$^7$ | 4       | Btn-GLMVGGV        | 500 ➔ 200 | LMVTGV LPVGGV | 20 ➔ 100 20 ➔ 100 | 60         | TBS-BC |
| 5     | 4.2 x 10$^7$ | 4       | Btn-GLMVGGV        | 500 ➔ 50  | LMVTGV LPVGGV | 100 100 | 60         | TBS-BC |
| 6     | 4 x 10$^7$   | 2       | Btn-GLMVGGV        | 100        | LMVTGV LPVGGV | 100 100 | 60         | TBS-BC |
| 7     | 2.5 x 10$^7$ | 2       | Btn-GLMVGGV        | 100 ➔ 50  | LMVTGV LPVGGV | 100 100 | 60         | TBS-BC |
| 8     | 1 x 10$^7$   | 5       | Btn-GLMVGGV        | 500 ➔ 50  | LMVTGV LPVGGV | 100 100 | 60         | TBS-BC |
| 9     | 7 x 10$^7$   | 6       | Btn-GLMVGGV        | 50         | ALAVGGS ALPPAGS LPVGGV | 10 ➔ 50 10 ➔ 50 100 | 60 ➔ 30 | TBS-BC |
| 10    | 2 x 10$^7$   | 5       | Btn-GLMVGGV        | 5000 ➔ 500 | -                  | -           | 60         | PC      |
| 11    | 8 x 10$^6$   | 5       | Btn-GLMVGGV        | 500 ➔ 50  | -                  | -           | 60         | PC      |
| 12    | 2 x 10$^7$   | 5       | Btn-GLMVGGV        | 50         | -                  | -           | 60 ➔ 20   | PC      |
| 13    | 5 x 10$^7$   | 6       | Btn-GLMVGGV        | 50 ➔ 30   | -                  | -           | 20         | PC      |
| 14    | 4.4 x 10$^7$ | 6       | Btn-GLMVGGV        | 30         | -                  | -           | 20         | PC      |
| 15    | 1 x 10$^7$   | 5       | Btn-Aβ40           | 200 ➔ 20  | -                  | -           | 60 ➔ 15   | PC      |
| 16    | 1.2 x 10$^7$ | 6       | Btn-Aβ40 Bn-Aβ42   | 10 ➔ 5    | -                  | -           | 15         | PC      |

TBS-BC = 100 mM Tris pH 7.5, 500 mM NaCl, 1% BSA, 5 mM CaCl$_2$. PC = human plasma, 5 mM CaCl$_2$. 
Searching publicly available databases and manufacturer’s catalogs for ELISA kits that detect human Aβ reveals kits designed to be used with standards ranging in concentration from 7.8 to 100,000 pg/mL, with most individual kits ranging from ~10 to 1000 pg/mL. Most commercially available kits are designed for the detection of a single Aβ alloform, normally Aβ40 or Aβ42. In contrast, SrtAβ has been shown to modify Aβ40, Aβ42, and Aβ43. The performance of these kits can be measured in multiple ways. The lower limit of quantitation (LLoQ) is defined as the lowest standard concentration with a signal higher than the average signal of the blank samples plus nine standard deviations that allows a percent recovery of 80-120%. The lower limit of detection (LLoD) is defined as the lowest standard concentration with a signal higher than the average signal of the blank samples plus nine standard deviations. The minimum detectable dose (MDD), referred to as sensitivity by some manufacturers, is the lowest concentration of analyte that can be differentiated from zero. It is obtained by taking the average of the blanks, adding 2 (MDD + 2) or 3 (MDD + 3) standard deviations, and using that value to calculate a concentration. In six ELISA experiments on six separate days, we observed LLoQ = 39 pg/mL on five occasions and LLoQ = 78 pg/mL once. We observed LLoD = 39 pg/mL on all six occasions. The average MDD across assays was 5.5 or 7.2 pg/mL, depending on whether 2 or 3 standard deviations were added to the average of the blanks.

### Supplementary Table 2. Comparison of developed ELISA to commercial kits

| Source                 | Alloform(s) detected | Standard range (pg/mL) | LLoQ (pg/mL) | LLoD (pg/mL) | MDD + 3 (pg/mL) | MDD + 2 (pg/mL) |
|------------------------|----------------------|------------------------|--------------|--------------|----------------|-----------------|
| This Work              | 40 and longer        | 39-2500                | 39-78        | 39           | 7.2            | 5.5             |
| LifeSpan BioSciences   | 40                   | 12.4-1000              | -            | -            | <4.6           | -               |
| LifeSpan BioSciences   | 42                   | 15.6-1000              | -            | -            | <9.4           | -               |
| RayBiotech             | 40                   | 100-100000             | -            | -            | -              | 100             |
| Biomatik               | 40                   | 12.4-1000              | -            | -            | -              | 4.6             |
| Biomatik               | 42                   | 12.4-1000              | -            | -            | -              | 5               |
| R&D Systems            | 40                   | 15.6-1000              | -            | -            | -              | 4               |
| R&D Systems            | 42                   | 7.8-500                | -            | -            | -              | 2.3             |
| Biorbyt                | 40                   | 125-8000               | -            | -            | 31.2           | -               |
| Biorbyt                | 42                   | 312-20000              | -            | -            | 78             | -               |
| IBL International      | 40                   | 188-1880               | -            | -            | 104            | -               |
| IBL International      | 42                   | 7.8-125                | 28.6         | 16           | -              | -               |
| Abexa                  | 40                   | 15.6-1000              | -            | -            | 9.4            | -               |
| Abexa                  | 42                   | 15.6-1000              | -            | -            | 9.4            | -               |
| Thermo                 | 40                   | 7.8-500                | -            | -            | -              | <6              |
| Thermo                 | 42                   | 15.6-1000              | -            | -            | <10            | -               |
| Novus                  | 40                   | 15.6-1000              | -            | -            | 9.4            | -               |
| Novus                  | 42                   | 15.6-1000              | -            | -            | 9.4            | -               |
| Mutant | Forward Primer | Reverse Primer |
|--------|----------------|----------------|
| L76I   | GCCGGGCTATATTGAAATTC | ACTTTGCTTTTATCTTTTCG |
| C102S  | ACCGTGGCG/ideoxyU/GTCTTTTGTG | ACGCCACGGG/ideoxyU/CGAGCTGTC |
| D105E  | AAGACGAAAGCC/ideoxyU/GGATGATCAG | AGGGCTTTCTGCT/ideoxyU/CTTCCACAAGCACAGCC |
| D107N  | GAAAGCCTGGATGA/ideoxyU/CGAAGAC | ATCATCCAGGCTT/ideoxyU/CGTTTTCTGACCACAAGC |
| I118S  | GTCTACCAGCGGCT/ideoxyU/CCTTGGT | AAGCCGGGT/ideoxyU/GACCCGAAATGCAATGTGTCGATCATCAGG |
| L123I  | ACTATCGATT/ideoxyU/ACCAACCTGAG | GTAAACTGATAG/ideoxyU/GCCCGACGAGACTGCG |
| L124D  | TACCAGCGCTTACGCTCACT | TGACGGATAATGCTATTGCTT |
| H127N  | AACTATCGATT/ideoxyU/ACCAACCTGAG | AAATGTGAGT/ideoxyU/GGGGACAAGAGCAGCG |
| R134G  | CGAACTAGACAGCA/ideoxyU/GCTCTG | GATCGTGCTCTATGTT/ideoxyU/GCCCGGTTCCAGTGC |
| L138K  | GCCGGGCGAAAAAGACAGCATCG | CTCAGGGTGGATACTGAGT |
| D139G  | AGCATTGCGTGCTATT/ideoxyU/ACAGTG | GTATAATACAGATC/ideoxyU/GACCTGTTTCGCCG |
| I141M  | ACTAGACAGCGTGGTGTTTTACGATT | TCCGGCCGCTCGAGT |
| T145K  | ATCTGTGATTATTAAAG/ideoxyU/GGCAACG | ACTTTAAAAACAGA/ideoxyU/GCTGTCTAGTTTCG |
| R152K  | CGAAACCGTAAGTATAAAAAAACAGCG | TTGCCCACGTGAAAATAC |
| I155M  | CCACTGTTGTAAGC/ideoxyU/GAGAC | ACGTTCAAGAATGC/ideoxyU/GGTCTTTATATCTACGGGTTC |
| C159R  | AGCATTGCGTGCCG/ideoxyU/GAGACCAGCG | ACGTTACAGATGC/ideoxyU/GGTATTTTTATCTACGG |
| R162K  | ACCGCGGTTCGAAG/ideoxyU/GCTGGATC | CACTTCCACGCGGAT/ideoxyU/GCGTTTCACGGTTACCA |
| H172Q  | AGGAAAGCAAAGA/ideoxyU/GAGCACG | ATCTTTGGCTTTCC/ideoxyU/GTTCATCCAGCACCACCTCC |
| E173K  | ATAAAGGCAAAGA/ideoxyU/GAGACAGCTGAC | ATCTTTGGCTTTTA/ideoxyU/GTTCATCCAGCACTCCAC |
| R177K  | AGGCAAAGATAACAGCTGACC | TCAATTTCCAGCAGCCATCTCC |
| A182V  | ACCTGCATGAT/ideoxyU/ATAACTATG | AATCATCAGCCGACCGCTGTC |
| Y189V  | AAAACCGGCGTGT/ideoxyU/GGGAATCCAG | ACAGCGGCTGGT/ideoxyU/CTACGTATTGATCATGCGAGTG |
| S196T  | CGGTGTGGGAAAHTAGTTAAATTCTTG | CGGGGTTTACAGTTAATC |
| S197R  | GTGGGAATCCCGTAAAAATTGTG | ACGCCCTTTCTAGTTAATC |
| R206K  | ACCGAAGTGAAAGGA/ideoxyU/GGCACAA | ATCTTTTCAGTCCGGT/ideoxyU/GCCAC |

**Supplementary Table 3. Primers used to generate reversion mutants.**
| Sample | Sex | Age (years) |
|--------|-----|-------------|
| 1      | F   | 78          |
| 2      | M   | 61          |
| 3      | M   | 64          |
| 4      | F   | 86          |
| 5      | M   | 73          |
| 6      | M   | 64          |
| 7      | M   | 67          |
| 8      | M   | 67          |
| 9      | M   | 72          |
| 10     | F   | 78          |

Supplementary Table 4. Sex and age of cerebrospinal fluid donors.
Supplementary Figure 1. TEV treatment reduces SrtA display to uninduced levels. TEV cleavage to remove SrtA also removes its C-terminal c-Myc tag. Staining of an induced population of cells, cells from that same population that have been treated with TEV as described above, and uninduced cells for c-Myc tag (chicken anti-c-myc, Invitrogen A-21281, followed by goat anti-chicken IgY AlexaFluor 488 conjugate, Invitrogen A-11039) shows that TEV treatment reduces the amount of apparent c-Myc to uninduced levels.
Supplementary Figure 2. Evolved sortase activity on fetuin A. (a) Fetuin A (5 μM) was incubated with SrtA 8.5-H3 (20 μM) and GGGK(Btn) (100 μM). Labeled fetuin A is detected by Western blot. The laddering in lane 4 is only observed in the presence of both SrtA and fetuin. (b) Western blot of overnight reaction of SrtA 8.5-H3 (50 μM) and GGGK(Btn) (1 mM) in human plasma shows labeling of endogenous fetuin A. (c) In a two hour reaction of SrtAβ (1 μM) and GGGK(Btn) (1 mM) in human plasma, no enzyme dependent modifications are observed upon streptavidin pulldown and Coomassie staining. The bands observed in the +SrtAβ +GGGK(Btn) lane are also observed in the GGGK(Btn) only lane. Notably, treatment with sortase 4S.6 under the same conditions leads to pulldown of a protein not observed in the other lanes. (d) Western blot of these reactions prior to pulldown shows that 4S.6, but not SrtAβ, labels fetuin A. This is notable evidence of a change in substrate specificity between 4S.6 and SrtAβ. Labeling of purified fetuin A, plasma Western blot, and plasma pulldown were each performed three times with similar results.
Supplementary Figure 3. Mutational analysis of the round 7 consensus sequence.
Single mutant reversions from the round 7 consensus sequence (R7) were made back to 4S.6 and wild-type SrtA. After induction and preparation for cell surface sortase reactions, clonal populations were incubated for 1 hour with 100-1000 nM Btn-LMVGG. Reversion mutant Y94R showed a 2.3-fold improvement on average across substrate concentrations compared to the round 7 consensus sequence. L123I (1.5-fold) and L124D (1.1-fold) also showed improvement. These three residues and residue 122 (by virtue of its proximity to 123 and 124) were targeted for site-saturation mutagenesis heading into round 9. Activity is defined as fold-increase in PE signal over a negative control (0 nM Btn-LMVGG) aliquot of each variant.
Supplementary Figure 4. Calcium dependence of evolved SrtAβ. Several mutations (green) in SrtAβ map near the calcium (orange) binding site. To assess the impact of these mutations on sortase calcium dependence, we treated 20 μM Abz-LMVGG(Dnp)-CONH₂ with SrtAβ in the presence of varying concentrations of calcium. Samples containing calcium showed an increase in fluorescence over time, while samples lacking calcium failed to rise above the level of a negative control lacking enzyme. Notably, SrtAβ shows activity at physiologically relevant calcium concentrations (typical ionized calcium levels in plasma range from 1.3-1.5 mM).
Supplementary Figure 5. HPLC traces of semi-syntheses and other reaction mixtures.
(a) In a representative injection from the AβM1-37GGGK(Btn) semi-synthesis described
above, 82% of the starting AβM1-40 was converted to the desired product, with no clear evidence of hydrolysis or alternate transpeptidation products. (b) 120 μM chemically synthesized Aβ40 (0.25 mg scale) was reacted overnight with 40 μM SrtAβ and 1 mM of the indicated glycine-based nucleophile before lyophilization, dissolution in 7 M guanidium chloride, 50 mM Tris pH 7.5, 2 mM EDTA, and analysis of the crude reaction mixture by HPLC. In the presence of SrtAβ but the absence of glycine nucleophiles, we observed a peak that does not fully resolve from the enzyme or Aβ40. This putative hydrolysis product has an area roughly one-quarter that of the Aβ40 peak. In the presence of glycine nucleophiles this product is never observed. Instead, we observe the expected transpeptidation products in yields of 80-88%. (c) In a representative injection from the AβM1-37GGGRR semi-synthesis described above, 64% of the starting AβM1-42 was converted to the desired product, with no clear evidence of hydrolysis or alternate transpeptidation products. (d) 120 μM chemically synthesized Aβ42 (0.4 mg scale) was reacted overnight with 30 μM SrtAβ and 1 mM or 200 μM GGGRR before lyophilization, dissolution in 7 M guanidium chloride, 50 mM Tris pH 7.5, 2 mM EDTA, and analysis of the crude reaction mixture by HPLC using the column and protocol from the AβM1-37GGGK(Btn) semi-synthesis. 64% of the Aβ42 was converted to the expected product when reacted with 1 mM GGGRR as opposed to 50% when reacted with 200 μM GGGRR.