**Rapid Selection of Cyclic Peptides that Reduce α-Synuclein Toxicity in Yeast and Animal Models**

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## SUPPLEMENTARY METHODS

### Plasmid and library construction

Library construction was performed essentially as described[1,2]. CX7Lib3 and LibRev1 primers were used to amplify the SICLOPPS insert region from the HPQ plasmid while incorporating the sequence $TGT(NNS)_7$ to encode the CP library. The NNS codon (where N is any nucleotide and S is guanine or cytosine) was used for amino acid randomization to allow representation of all twenty amino acids while reducing the impact of degeneracy and stop codons. Zipper and LibRev1 primers were then used in a second PCR reaction that has been shown to minimize frameshifts[1,2]. This insert was then doubly digested with BglI and XbaI overnight and purified (PCR purification kit, QIAgen). A 2μ plasmid encoding the SICLOPPS HPQ construct downstream of a *PGK* promoter was also doubly digested with BglI and XbaI overnight, then further digested with KpnI which has a unique site within the HPQ-encoding motif to help prevent vector re-ligation. The cut vector was gel purified and ethanol precipitated. Ligation conditions were optimized, and ten ligations using 400 ng vector and 240 ng insert were performed overnight at 16 °C, ethanol precipitated, and transformed via electroporation into electrocompetent *E. coli*. Transformants from ten electroporations were pooled and plated onto ten large library plates (10 cm x 10 cm) of LB-Amp and incubated at 37 °C overnight. Serial dilutions were also plated to measure overall transformation efficiency. Colonies were scraped, suspended in 25% glycerol and frozen. 400 µL of frozen stock was used to prepare 100-200 µg DNA (Maxiprep kit, QIAgen).

Mutagenesis and biological analysis of the effects of CP1 and CP2 were performed using CEN plasmids to ensure moderate and reproducible expression levels. These were generated by subjecting the plasmids isolated from the library to PCR with primers CBDRev2 and IcFwd1, then cloning the amplified inserts into a Gateway entry vector using TOPO cloning (Invitrogen). Then LR Clonase reactions were used with the destination vector pAG415GPD to generate new expression vectors[3]. This vector differs from the original library vector in three ways: the SICLOPPS construct is expressed off a different constitutive promoter (the *GPD* promoter), the auxotrophic marker is *LEU2* rather than *URA3*,

and the plasmids are low-copy CEN plasmids rather than high-copy 2μ plasmids. Despite these changes, CP1 and CP2 retained similar activity in α-syn-expressing model strains. All mutagenesis was performed using standard site-directed mutagenesis techniques (Quikchange mutagenesis, Stratagene, and similar strategies). Fluorescence and electron microscopy of yeast expressing α-syn and CP contructs was performed as described[4].

We designed the mCP gene to encode the SICLOPPS construct followed by an HA tag and flanked by Gateway cloning sites, and the gene was synthesized from component oligonucleotides (GeneArt, Inc.). CP1 and CP2 sequences and splice-disabling mutations were introduced using site-directed mutagenesis. LR Clonase reactions were used to transfer the mCP genes to the worm DA-specific expression plasmid pDEST-DAT-1. GFP was introduced by cloning in a GFP cassette between PstI and EcoRI sites downstream of the HA tag.

**Preparation of Affinity Reagents**

Head-to-tail cyclized peptides were synthesized using standard Fmoc solid-phase chemistry, cyclized prior to side chain deprotection, and purified to 95% by RP-HPLC (Cambridge Peptides, Inc.). MALDI-TOF mass spectrometry was used to confirm the identities of the cyclic peptides ($MW_{(M+H)}$=905.2, $MW_{obs}$=905.5 for $CP1_{R7K}$, $MW_{(M+H)}$=917.2, $MW_{obs}$=917.6 for $CP2_{W7K}$). $CP1_{R7K}$ and $CP2_{W7K}$ (**Supplementary Fig. 4**) were reacted with Aminolink Plus Coupling Resin (Pierce) according to manufacturer's instructions. UV spectroscopy indicated that resin was loaded to 1-2 mg per mL of gel bed volume. $CP1_{R7K}$ and $CP2_{W7K}$ were also reacted with NHS-LC-Biotin (Pierce) and Sulfo-SBED Biotin Label Transfer Reagent (Pierce) to geneate biotinylated and photo-activatable crosslinking analogs rapidly and with high efficiency.

**Generation of CP1-GFP and CP2-GFP transgenic worms**

mCP_CP1-GFP and mCP_CP2-GFP were cloned into pDEST-DAT-1 via Gateway technology (Invitrogen, Carlsbad, CA) to selectively express the transgenes in the worm DA neurons under the control of *dat-1* promoter. The expression plasmids along with a marker *rol-6* (50 μg/ml each plasmid) were directly microinjected into the gonads of wildtype N2 strain to generate UA116 [*baEx91*; $P_{dat\text{-}1}$::mCP1_CP1-GFP; *rol-6* (*su1006*)] and UA117 [*baEx92*; $P_{dat\text{-}1}$::mCP_CP2-GFP; *rol-6* (*su1006*)]. Three independent transgenic worms were generated for each strain. Fluorescence microscopy was performed to verify the expression of CPs as described previously[5].

**SUPPLEMENTARY DATA**

**Co-expression of selected CPs with established genetic suppressors of α-syn toxicity**

Two important genetic suppressors of α-syn toxicity uncovered using our yeast model are Ypt1 and Ypk1. Ypt1 is a Rab GTPase involved in ER-to-Golgi transport and Ypk9 is a P-type ATPase with homology to the human *PARK9*/ATP13A2 gene which is linked to an early-onset form of Parkinsonism. Homologues of both these proteins have been shown to reduce α-syn toxicity in metazoan models including the nematode model[4,6,7]. To determine whether CP1 and CP2 act in different pathways from Ypt1 and Ypk9, we expressed them along with the genetic suppressors in a higher-toxicity α-syn strain[4,7]. Genetic suppressors were cloned into the pAG413GPD yeast expression vector and the CPs were expressed using pAG425GPD-derived constructs[3] (**Supplementary Fig. 5**).

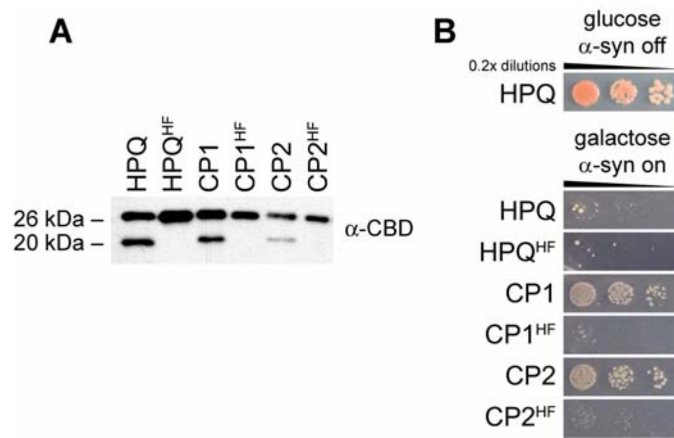
**Block F mutants and constructs encoding cleavage products further confirm that CP1 and CP2 require complete splicing for activity**

The T69A/H72A double mutation, which resides in conserved block B of the *Ssp. dnaE* N-intein domain, was used as a convenient splice-disabling mutation. This mutant is predicted to be deficient in processing at the extein/N-intein junction, which is an obligatory first step in *dnaE* split intein splicing [8,9]. In some circumstances, interfering with N-intein processing can lead to cleavage at the extein/N-intein junction without concomitant processing at the extein/C-terminal junction [8,10]. In the SICLOPPS construct, this would represent a cleavage event that would leave the encoded peptide as a linear fusion with the C-intein domain. Blotting for the chitin binding domain tag appended to the N-intein would thus not distinguish this cleavage event from complete splicing.

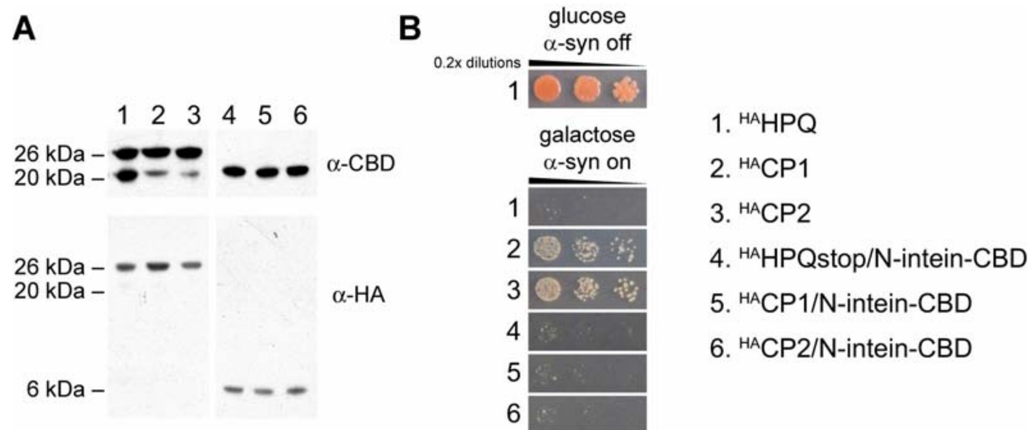
To ensure that complete splicing is indeed necessary for the function of the CP1 and CP2 constructs, we performed two controls in addition to testing the block B mutants. The first involved making a different mutation that interferes with C-intein processing to verify that C-intein processing is also required for function. By analogy with structural and functional studies on similar inteins, H24 and F26 in conserved block F of the *dnaE* C-intein domain are responsible for promoting asparagine cyclization in the final processing step at the extein/C-intein junction[9,11]. Previous work using the SICLOPPS construct has verified that mutation of these residues abolishes splicing *in vitro*[10,12]. Point mutagenesis on plasmids encoding HPQ, CP1 and CP2 constructs was used to generate H24L/F26A double mutants, and the resulting plasmids were sequenced to verify the mutation. These H24L/F26A mutants express in yeast but fail to cleave or splice as evident by the presence of only the full-length SICLOPPS construct (**Supplementary Fig. 1a**). Further, they fail to suppress α-syn toxicity in yeast (**Supplementary Fig. 1b**).

A second set of experiments was undertaken to directly test the possibility that a cleavage event that produces a linear peptide-C-intein fusion is required for CP1 and CP2 function (rather than a full splicing event). To replicate the results of a possible N-intein cleavage event, we decided to co-express the N-intein domain along with a peptide-C-intein fusion. First, to enable detection of expressed peptide-C-intein fusions, we introduced an HA tag to the N-termini of the HPQ, CP1 and CP2 constructs using insertion mutagenesis. These constructs were verified to express and cleave/splice by blotting for either the C-intein HA tag or the N-intein CBD tag (**Supplementary Fig. 2a**). The HA tag did not alter the abilities of the CP1 and CP2 constructs to reduce α-syn toxicity (**Supplementary Fig. 2b**). Next, we introduced stop codons immediately following the encoded peptide regions of the HA-tagged HPQ, CP1 and CP2 constructs to generate the peptide-C-intein fusion genes. We also cloned the N-intein-CBD fusion from the SICLOPPS construct using Gateway-compatible PCR primers and then used a BP clonase reaction to generate an N-intein-CBD entry vector. This entry vector was used to make a 2μ expression vector using pAG425GPD-*ccdB* as described[3]. All vectors were sequenced to ensure the presence of the appropriate insertions, mutations, and positioning within newly cloned vectors. Robust expression of each component was verified by blotting for the appropriate tag (**Supplementary Fig. 2a**). Expression of the HA-tagged peptide-C-intein fusions alone or with the N-intein-CBD fusion failed to suppress α-syn toxicity (**Supplementary Fig. 2b**). Thus, the products of a hypothesized N-intein cleavage event do not phenocopy the full, splice-competent SICLOPPS constructs encoding CP1 and CP2. This control, when combined with the requirements for N-intein processing and C-intein processing demonstrated using point mutants, shows that CP1 and CP2 splice *in vivo* to produce cyclic peptides that are responsible for the observed phenotypes.

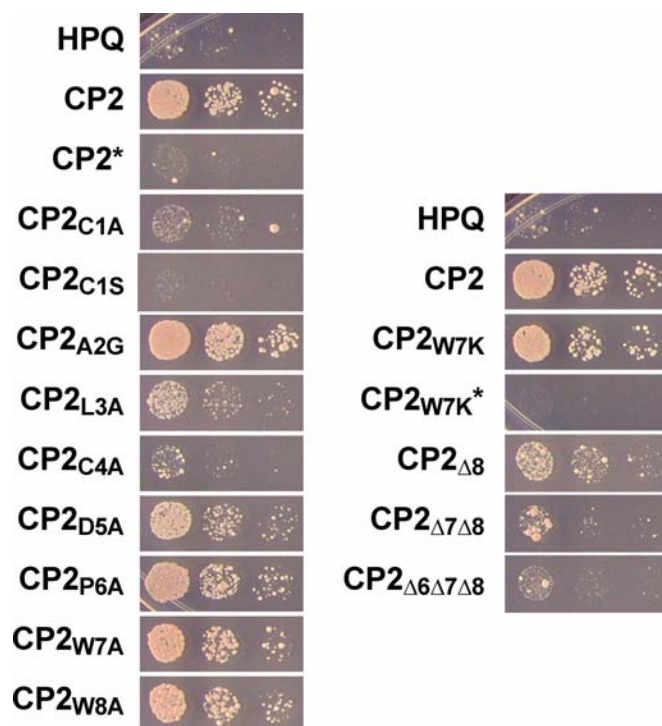
## SUPPLEMENTARY FIGURES



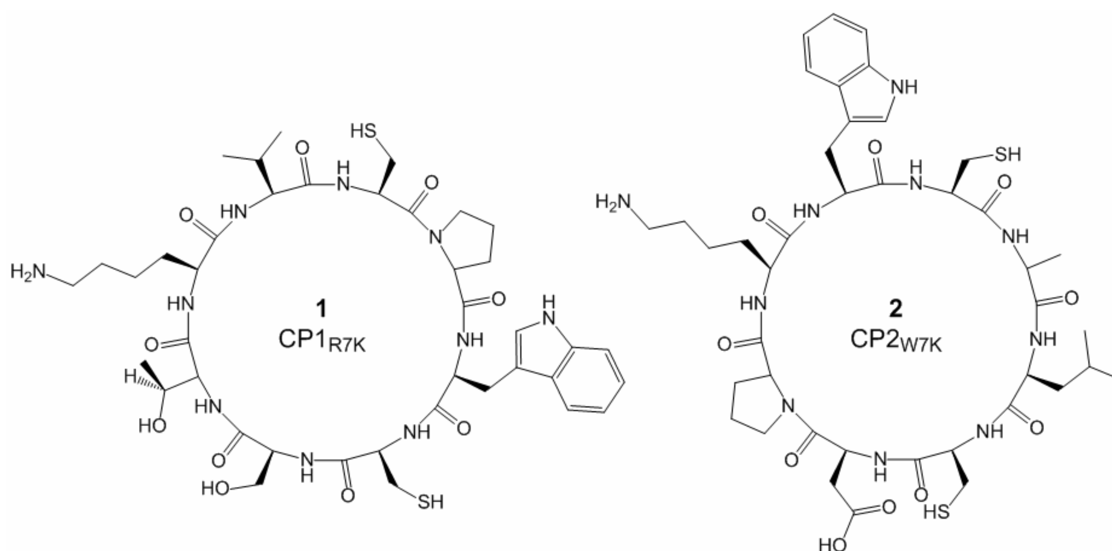

**Supplementary Figure 1. Mutations that interfere with C-intein processing abolish the activities of CP1 and CP2**. (A) Western blots using anti-CBD serum show that H24L/F26A mutants of HPQ, CP1 and CP2 constructs ($HPQ^{HF}$, $CP1^{HF}$, and $CP2^{HF}$) express but do not splice in yeast. (B) Spotting assays similar to Figure 2B demonstrate that H24L/F26A mutants of HPQ, CP1 and CP2 constructs do not suppress α-syn toxicity.



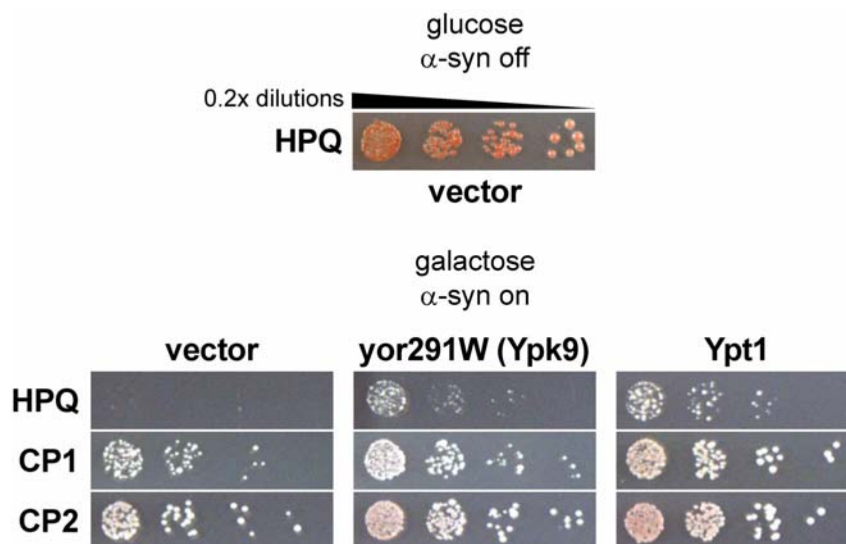

**Supplementary Figure 2. Direct expression of products of a hypothetical intein cleavage event do not suppress α-syn toxicity**. (A) Western blots using anti-CBD (top) and anti-HA (bottom) antibodies demonstrate that HA-tagged HPQ, CP1 and CP2 constructs ($^{HA}$HPQ, $^{HA}$CP1, $^{HA}$CP2) express and process in yeast. Further, HA-tagged constructs with stop codons immediately following the CP-encoding region ($^{HA}$HPQstop, $^{HA}$CP1stop, $^{HA}$CP2stop) also express in yeast. These latter constructs were co-expressed with a heterologous N-intein-CBD fusion construct to ensure both products of the hypothetical intein cleavage event were present. (B) Spotting assays similar to those shown in **Fig. 2b** demonstrate that the products of intein cleavage do not suppress α-syn toxicity.



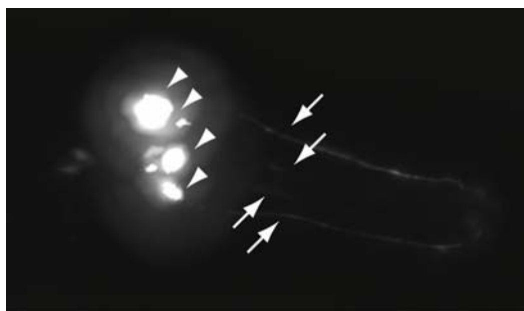

**Supplementary Figure 3. SAR and minimization data of CP2.** Spotting assays of normalized, serially diluted cells of the screening strain expressing CP2, indicated mutants of CP2, and the negative control HPQ. Each construct was verified to express and splice in yeast. Asterisks denote splice-disabled mutants, which were verified to express but not splice. Similar results were obtained for CP1 (**Fig. 3**).




**Supplementary Figure 4. Chemical structures of CP1R7K (1) and CP2W7K (2).**




**Supplementary Figure 5. Co-expression of selected CPs with Ypt1 and Ypk9.** Ypt1 and Ypk9 (yor291w) were expressed using a pAG413GPD vector and CP constructs HPQ, CP1 and CP2 were expressed using a pAS425GPD vector[3].



**Supplementary Figure 6. *C. elegans* express mCP-GFP constructs in dopaminergic neurons**. The re-optimized mCP gene was cloned downstream of the *dat-1* promoter and injected into wild-type worms. The mCP-GFP fusion was transiently expressed in DA neurons and showed robust GFP fluorescence. This representative fluorescence micrograph shows a 7-day-old worm with robust expression of mCP-GFP, with cell bodies (arrowheads) and processes (arrows) visible. Note that not all DA neurons are expected to express mCP-GFP due to mosaic expression of transgenes in non-integrated transgenic animals, and that these effects are averaged out by the large number of animals and multiple transgenic lines used in generating data on α-syn-related phenotypes; this is consistent with methodology used in prior studies[4-7].

## SUPPLEMENTARY TABLE 1 – SEQUENCE DATA

**CP Sequence Data**

| Name | CP Sequence | DNA Sequence |
|---|---|---|
| CP1 | CPWCSTRV | TGCTCGACGAGGGTGTGCTTAAGT |
| CP2 | CALCDPWW | TGTGCGTTGTGCGACCCCTGGTGG |
| CP3 | CFGRYWFC | TGTTTCGGCCGGTACTGGTTCTGC |
| CP4 | CLATWAVG | TGTCTGGCCACCTGGGCGGTCGGC |
| | | |
| CX7Lib-1 | CRWLGVEG | TGTCGGTGGTTGGGGGTGGAGGGG |
| CX7Lib-2 | CFTNVHPQFANA | TGTTCACCAACGTCCACCNGCAGTTCGCGAACGCG |
| CX7Lib-3 | frameshift | TGTTCCGCGGGCACGTTGAGCTGC |
| CX7Lib-4 | frameshift | TGTGGCTAGCGGGAGGGGGTGTTG |
| CX7Lib-5 | CRLSGAGL | TGTCGNTTGTCCGGCGCGGGGTTG |
| CX7Lib-6 | CVAGERRR | TGTGTGGCCGGGGAGCGGCGGCGC |
| CX7Lib-7 | CAGGWWGA | TGTGCCGGGGGGTGGTGGGGGGCG |
| CX7Lib-8 | CKG-MGLM | TGTAAGGGGTAGATGGGGTTGATG |
| CX7Lib-9 | CGICSEWA | TGTGGGATCTGCTCCGAGTGGGCG |
| CX7Lib-10 | CEGDVMWA | TGTGAGGGCGACGTGATGTGGGCG |
| CX7Lib-11 | CSKGDGWL | TGTTCGAAGGGGGACGGCTGGCTG |
| CX7Lib-12 | CACTRPAS | TGTGCCTGCACCCGCCCGGCGTCG |
| CX7Lib-13 | CGALVLWM | TGTGGCGCGTTGGTGTTGTGGATG |
| CX7Lib-14 | frameshift | TGTGCCGTGGCCGTCTGCAACACG |
| CX7Lib-15 | CAARGRVR | TGTGCGGCCAGGGGGAGGGTGAGG |
| CX7Lib-16 | CRCWVERL | TGTCGGTGCTGGGTGGAGCGGTTG |
| CX7Lib-17 | CVGRERWA | TGTGTGGGNCGCGAGAGGTGGGCG |
| CX7Lib-18 | CWNSCGCG | TGTTGGAACAGCTGCGGGTGCGGG |
| CX7Lib-19 | CCGRSRMR | TNTTGCGGGCGGTCGCGGATGAGG |
| CX7Lib-20 | CWLGVRVA | TGTTGGCTCGGCGTGCGCGTGGCG |

**SICLOPPS Sequence**

| Name | CP Sequence | DNA Sequence |
|---|---|---|
| HPQ | CFTNVHPQFANA | ATGGTTAAAGTTATCGGTCGTCGTTCCCTCGGAGT<br>GCAAAGAATATTTGATATTGGTCTTCCCCAAGACC<br>ATAATTTTCTGCTAGCCAATGGGGCGATCGCCCAC<br>AATTGTTTCACCAACGTCCACCCGCAGTTCGCGAA<br>CGCGTGCTTAAGTTTTGGCACCGAAATTTTAACCG<br>TTGAGTACGGCCCATTGCCCATTGGCAAAATTGTG<br>AGTGAAGAAATTAATTGTTCTGTGTACAGTGTTGA<br>TCCAGAAGGGAGAGTTTACACCCAGGCGATCGCCC<br>AATGGCATGACCGGGGAGAGCAGGAAGTATTGGAA<br>TATGAATTGGAAGATGGCTCAGTAATCCGAGCTAC<br>CTCTGACCACCGCTTTTTAACCACCGATTATCAAC<br>TGTTGGCGATCGAAGAAATTTTTGCTAGGCAACTG<br>GACTTGTTGACTTTAGAAAATATTAAGCAAACTGA<br>AGAAGCTCTTGACAACCATCGTCTTCCCTTTCCAT<br>TACTTGACGCTGGTACCATTAAAACGACAAATCCT<br>GGTGTATCCGCTTGGCAGGTCAACACAGCTTATAC<br>TGCGGGACAATTGGTCACATATAACGGCAAGACGT<br>ATAAATGTTTGCAGCCCCACACCTCCCTGGCAGGA<br>TGGGAACCATCCAACGTTCCTGCCTTGTGGCAGCT<br>TCAATGA |

## SUPPLEMENTARY TABLE 2 – PRIMERS

**Primers for Plasmid and Library Construction**

| Name | Sequence | Use |
|---|---|---|
| LibRev1 | CATGATGCGGCCCTCTAGGATCAGCG | Constant primer for library PCR |
| Zipper | GGAATTCGCCAATGGGGCGATCGCC | Constant primer for library PCR |
| CX7Lib3 | GGAATTCGCCAATGGGGCGATCGCCCACAATTGTNNSNNSNNSNNSNNSNNSNNSTGCTTAAGTTTTGGC | Randomized primer for library PCR, generating the 8-mer library |
| | [ A C G T]<br>N = [25-25-25-25]<br>S = [ 0-25-25-0 ] | |
| CBDRev2 | CCTCTAGACTCGAGTCATTGAAGCTGC | Reverse primer for PCR of SICLOPPS region |
| IcFwd1 | CACCATGTCTTTAAGGATCCAAATGGTTAAAGTTATCG | Forward primer for PCR of SICLOPPS region with CACC overhang for TOPO cloning into Gateway entry vector |
| Nint-TH1 | GCTCAGTAATCCGAGCTGCCTCTGACGCCCGCTTTTTAACC | Mutagenesis primer for Block B T69A/H72A double mutation |
| Nint-TH2 | GGTTAAAAAGCGGGCGTCAGAGGCAGCTCGGATTACTGAGC | Mutagenesis primer for Block B T69A/H72A double mutation |
| Cint-HF1 | GGTCTTCCCCAAGACCtTAATgcTCTGCTAGCCAATGGG | Mutagenesis primer for Block F H24L/F26A double mutation |
| Cint-HF2 | CCCATTGGCTAGCAGAgcATTAaGGTCTTGGGGAAGACC | Mutagenesis primer for Block F H24L/F26A double mutation |
| Cint-HA | AAACAATGTCTTTAAGGATCCAAATGTACCCTTACGACGTGCCCGACTACGCCGTTAAAGTTATCGGTCGTCGTTCCCT | Mutagenesis primer for HA tag insertion |
| Cint-HA | AGGGAACGACGACCGATAACTTTAACGGCGTAGTCGGGCACGTCGTAAGGGTACATTTGGATCCTTAAAGACATTGTTT | Mutagenesis primer for HA tag insertion |
| HPQstop1 | CAGTTCGCGAACGCGTGATTAAGTTTTGGCAC | Mutagenesis primer for stop codon mutation after HPQ sequence |
| HPQstop2 | GTGCCAAAACTTAATCACGCGTTCGCGAACTG | Mutagenesis primer for stop codon mutation after HPQ sequence |
| CP1stop1 | CTCGACGAGGGTGTGATTAAGTTTTGGCACC | Mutagenesis primer for stop codon mutation after CP1 sequence |
| CP1stop2 | GGTGCCAAAACTTAATCACACCCTCGTCGAG | Mutagenesis primer for stop codon mutation after CP1 sequence |
| CP2stop1 | GACCCCTGGTGGTGATTAAGTTTTGGCACC | Mutagenesis primer for stop codon mutation after CP2 sequence |
| CP2stop2 | GGTGCCAAAACTTAATCACCACCAGGGGTC | Mutagenesis primer for stop codon mutation after CP2 sequence |
| Nint-F1 | CAAAAAAGTTGGCATGTTAAGTTTTGGCACCGAAATTTTAACCG | PCR primer for N-intein amplification |
| CBD-R2 | AGAAAGTTGGGTATTGAAGCTGCCACAAGGCAGGAAC | PCR primer for N-intein amplification |
| attB1Fwd | GGGGACAACTTTGTACAAAAAAGTTGGCATG | Bridge primer for N-intein amplification enabling BP cloning |
| attB2Rev | GGGGACAACTTTGTACAAGAAAGTTGGGTA | Bridge primer for N-intein amplification enabling BP cloning |

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