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A New Yeast Recombineering Tool for *C. elegans* RNAi

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Abstract

Proprietary technologies have been developed to enable expedited and efficient recombination of plasmids over traditional techniques. Here, we introduce a new tool for using the endogenous *S. cerevisiae* machinery to modify the L4440 plasmid. Using this tool, we generated a plasmid for genetic depletion of *cup-14* and employed the modified plasmid with a *C. elegans* model of neurodegeneration restricting RNAi to the nervous system by the pan-neuronal expression of the dsRNA transporter, *sid-1*. We show that depletion of the *C. elegans* PLEKHM1 orthologue, *cup-14*, increased Aβ-induced neurodegeneration in glutamatergic neurons, suggesting that coordination of anterograde trafficking of endolysosomes with autophagosomes plays an important role in Aβ-mediated neurodegeneration.

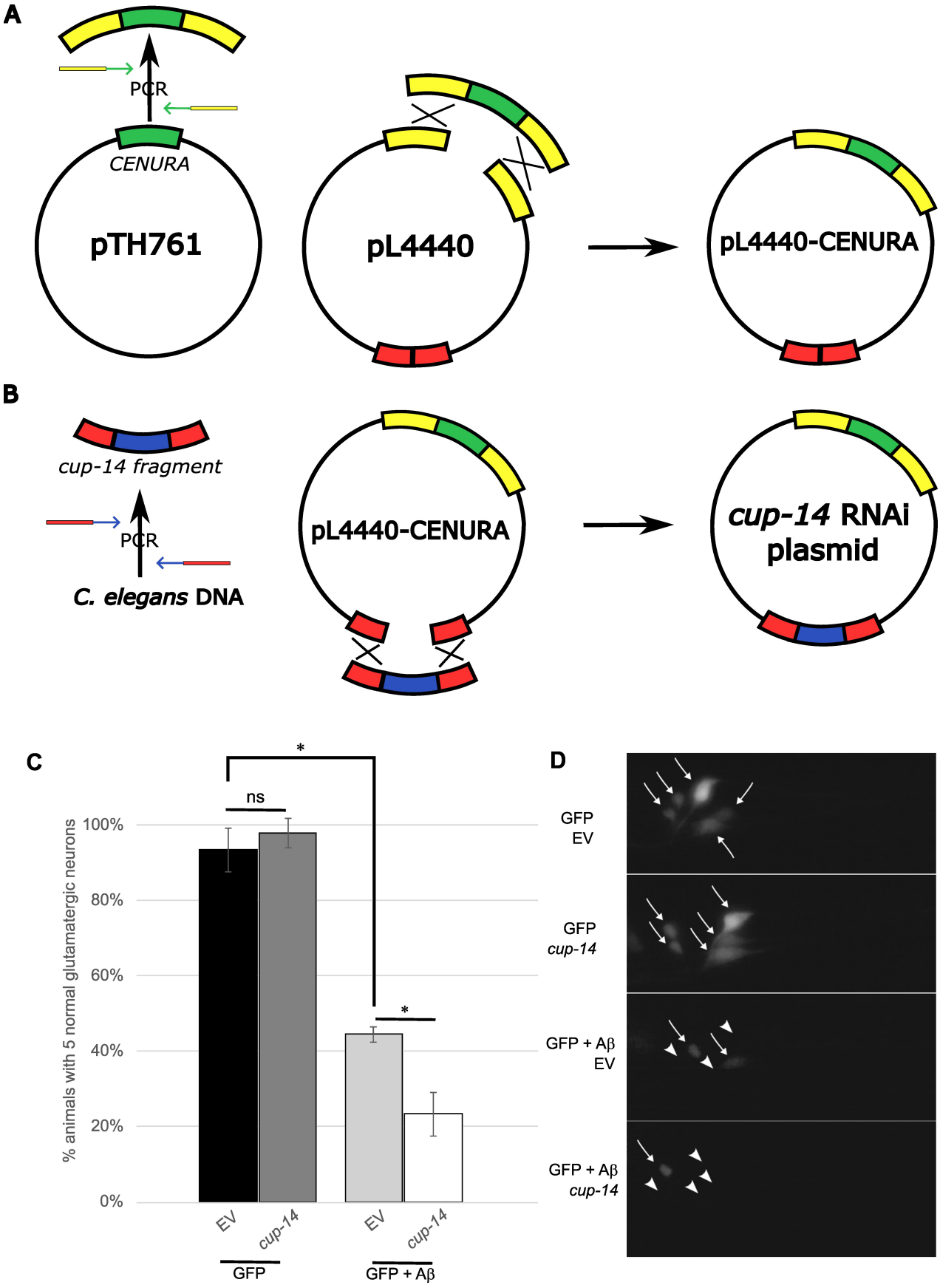


Figure 1. Cloning Schematic and Neurodegeneration Analysis:

(A) Schematic for cloning CENURA into pL4440 to create pL4440-CENURA. (B) Schematic for cloning genetic fragment of *cup-14* into pL4440-CENURA. (C) Depletion of *cup-14* by RNAi has no effect on GFP-only animals, but

significantly increases neurodegeneration of Aβ-expression animals compared to EV control. Data represented as mean (3 trials x 30 animals = 90 animals total) and error bars represent standard deviation. ns=not significant, *=p<0.05 according to One-Way ANOVA with a Tukey's *post-hoc* test. (D) Representative images of neurodegeneration. Arrows indicate normal neurons and arrowheads indicate neurons scored as degenerating.

Description

Homologous recombination in *Saccharomyces cerevisiae* is a powerful technique that has been utilized in bacterial applications (Shanks et al., 2006; Zegans et al., 2012). A double-stranded break in a plasmid vector capable of replication in yeast can be repaired by homologous recombination with a PCR-generated amplicon, which can then be selected in yeast and bacteria by selective markers in the vector backbone. The advantages of this technique are that it does not require proprietary reagents, multiple fragments can be integrated without additional PCR, and it does not require terminal restriction sites nor *in vitro* ligation. It is simple, efficient, and robust, such that it can be performed in the research laboratory or instructional laboratory. While it is more time consuming, hands-on experimental time is minimal. Though it requires more reagents, these reagents are much less expensive compared to proprietary reagents at scale. Here, we report a new application of yeast recombination for the generation of *C. elegans* RNAi constructs and demonstrate its utility in a *C. elegans* model of neurodegeneration with RNAi restricted to the nervous system.

To accomplish RNAi by feeding, the L4440 plasmid has two convergent T7 promoters, separated by a multicloning site, for the expression of dsRNA in HT115 *E. coli* fed to *C. elegans* (Timmons et al., 2001). However, for effective selection in uracil auxotrophic *S. cerevisiae*, a plasmid requires a centromere for inheritance and selection by the URA3 gene. These are adjacent to each other as a segment (CENURA) of pTH761 (Addgene). The CENURA fragment was amplified from pTH761 by PCR using primers with 5' homology to the designated site of insertion in pL4440, recombined with linearized pL4440 in *S. cerevisiae*, and selected by uracil depletion to generate a modified L4440 plasmid (pL4440-CENURA, Figure 1A). Using the same method, we then used pL4440-CENURA to generate a construct for depletion of a target gene in an *C. elegans* model of neurodegenerative disease with RNAi restricted to the nervous system.

Alzheimer's disease (AD) is characterized by the accumulation of insoluble plaques comprised of amyloid-β (Aβ) in the brain as a result of sequential cleavage of Amyloid Precursor Protein (APP). The Aβ can be internalized by endocytosis into neurons, whereupon it enters the endolysosomal system. Previously, we showed that over-expression of Aβ in the glutamatergic neurons of *C. elegans* induced neurodegeneration, which was mitigated by over-expression of human Vps41 (hVps41). This neuroprotective effect was dependent on genetic interactions between *arl-8*, which is responsible for anterograde trafficking of endosomal components, and autophagy (Griffin et al., 2018), and not *rab-7*, which regulates retrograde endolysosomal trafficking. Interactions between anterograde trafficking components and autophagy are mediated by direct interactions with the scaffolding protein PLEKHM1 (Griffin et al., 2019; Jia et al., 2017; Marwaha et al., 2017). Using this new recombineering tool, we recombined a fragment of the *C. elegans* PLEKHM1 orthologue, *cup-14*, into pL4440-CENURA for RNAi by feeding (Figure 1B). Using a *C. elegans* model expressing Aβ in the glutamatergic neurons and restricting RNAi to the nervous system (Griffin et al., 2018), we found that depletion of *cup-14* significantly increased neurodegeneration in Aβ-expressing animals (p=0.002) (Figure 1C and D).

While the role of extracellular deposition of Aβ remains unclear, it is evident that disruptions in the mechanisms coordinating autophagy with endolysosomal trafficking exacerbate Aβ toxicity. Previous work has implicated autophagosomal secretory mechanisms in Aβ toxicity (Nilsson et al., 2013) and here we show that disrupting coordination between anterograde endolysosomal trafficking and autophagosomes exacerbates Aβ toxicity. In contrast, over-expression of PLEKHM1 in mouse brains increased α-synuclein toxicity associated with Parkinson's disease (Höfs et al., 2025), while differential tissue expression levels appear to be associated with either decreased or increased risk of Parkinson's disease (Gokuladhas et al., 2025). Considering the distinct functional roles of hVps41 observed between α-synuclein and Aβ toxicity models (Griffin et al., 2018), there may also be a distinct role of PLEKHM1 between Parkinson's and Alzheimer's diseases as well.

Furthermore, we demonstrate the utility of exploiting the native recombination machinery of *S. cerevisiae*. Though this tool was originally employed in the construction of expression vectors for use in Gram-negative bacteria (Shanks et al., 2006), this vector applies those principles to include the efficient generation of constructs for RNAi by feeding in *C. elegans*.

Methods

C. elegans strains

C. elegans were maintained following standard procedures (Brenner, 1974). The strains UA338 *baIn34*[P_{eat-4}::Aβ, P_{myo-2}::mCherry]; *adIs1240*[P_{eat-4}::GFP]; *sid-1*(*pk3321*); *uIs69*[P_{myo-2}::mCherry; $P_{unc-119}$::*sid-1*] and UA339 *adIs1240*[P_{eat-4}::GFP]; *sid-1*(*pk3321*); *uIs69*[P_{myo-2}::mCherry; $P_{unc-119}$::*sid-1*] were generous gifts of Drs. Guy and Kim Caldwell.

Plasmid Construction

PCR was performed with the primers 5'-GTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATG TCACCTGGGTCCTTTTCATCAC-3' and 5'-CCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCA CATGTGTATACATGCATTTACTTATAATACAG-3', using pTH761 (Addgene) as template, to generate a fragment containing a yeast centromere and URA3 cassette. Homologous recombination was performed in yeast as previously described, with few modifications (Shanks et al., 2006). Briefly, *S. cerevisiae* was used to inoculate 5 mL of liquid YPD (1% bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose) and grown overnight with shaking at 30℃. The culture was then used to inoculate 50 mL of YPD and incubated on a shaker at 200 rpm for 3-5 hours. Cells were harvested by centrifugation at 12,000 rpm for 10 seconds, and washed once with 1 mL of sterile H_2O, once with 1 mL of 100 mM lithium acetate (LiAc), again with 400 µL of 100 mM LiAc, then aliquoted into 50µL samples. The plasmid vector was linearized at the site of recombination, using PciI according to manufacturer guidelines (New England Biolabs). Single-stranded carrier DNA (Herring sperm DNA 2.0 mg/mL; Promega) was denatured by boiling and chilled immediately. The transformation mixture was prepared by mixing 25 µL of carrier DNA, 25 µL of amplified DNA fragment, and 5 µL of linearized plasmid DNA (0.1-10 µg) with 500 µL of Lazy Bones solution (40% PEG3350, 100 mM LiAc, 1 mM EDTA and 1 mM Tris pH 8.0). The transformation mixture was incubated for 30 minutes at 30℃, followed by a heat shock for 20-25 minutes in a 42 ℃ water bath. After centrifugation, the transformation mix was removed and the pellet was resuspended in sterile H_2O, which was pipetted onto YPD and selective media. Plasmid extraction from yeast was performed by using the Zymoprep™ Yeast Plasmid Miniprep II and used to transform competent DH5α. Colonies were selected and plasmids extracted using the ZymoPURE™ Plasmid Miniprep Kit. Candidates were confirmed by Nanopore Sequencing (Eurofins Genomics), confirming creation of pL4440-CENURA (pEFG7). Exon 11 of *cup-14* was amplified from *C. elegans* gDNA using the primers 5'-ggggatccacgcgtcacgtggctagccatggaaccggtggatccactagtCTGCGCCATC GACAGCAAATA-3' and 5'-gcagatctgatatcatcgatgaattcgagctccaccgcggtggcggccgcCTATGCGTGGATTCCGTATTAAAC-3'. The *cup-14* fragment generated by PCR was recombined into pEFG7 using the modified protocol previously described, with the modification that pEFG7 was linearized using XbaI (Thermo Scientific) in order to insert the fragment between the T7 promoters. Recombined plasmids were extracted from yeast colonies as previously described and used to transform competent DH5α. Candidates were confirmed by Sanger sequencing (Eurofins Genomics). Candidate pEFG7 and *cup-14* (pEFG8) RNAi plasmids were used to transform competent HT115 for dsRNA feeding *C. elegans*.

RNAi

RNAi feeding clones were cultivated initially on LB solid medium containing tetracycline (5 µg/ml) and ampicillin (100 µg/ml) and then individual colonies were grown overnight in liquid LB medium containing 100 µg/ml ampicillin. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to NGM agar to a final concentration of 100 µM and seeded with RNAi feeding clones. Induction of dsRNA occurred during a 14- to 18-h incubation at 20 °C. Adult hermaphrodites were allowed to lay eggs for 3 h on RNAi feeding clones to produce a synchronized population. Glutamatergic neurons of synchronized progeny were analyzed at least 2 days after hatching, as described.

Neuron Analysis

Populations were synchronized by a 3-hour egg-lay, as previously described (Griffin et al., 2018), and incubated at 20 °C. To analyze glutamatergic neurons, animals were immobilized using 3 mM levamisole on glass cover slips, inverted onto 2% agarose pads on microscope slides, and observed by fluorescent microscopy. An animal was scored as normal if all 5 glutamatergic neurons in the tail were present and without malformations such as apoptotic swelling, distention, axon breaks, separation of the soma, or loss of fluorescence. Each analysis was replicated at least three times with 30 animals per condition (N = 3; n = 30). Images were acquired using an Olympus BX41 equipped with a FITC filter cube and MoticamX camera driven by Motic Images Plus 3.1.

Reagents

Strain	Genotype	Source
UA338	*baIn34*[P_{eat-4}::Aβ, P_{myo-2}::mCherry]; *adIs1240*[P_{eat-4}::GFP]; *sid-1*(*pk3321*); *uIs69*[P_{myo-2}::mCherry; $P_{unc-119}$::*sid-1*]	Griffin, 2018
UA339	*adIs1240*[P_{eat-4}::GFP]; *sid-1*(*pk3321*); *uIs69*[P_{myo-2}::mCherry; $P_{unc-119}$::*sid-1*]	Griffin, 2018

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