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Expanding the Genetic Code of an Animal

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Supporting Information

ABSTRACT: Genetic code expansion, for the site-specific incorporation of unnatural amino acids into proteins, is currently limited to cultured cells and unicellular organisms. Here we expand the genetic code of a multicellular animal, the nematode *Caenorhabditis elegans*.

Genetic code expansion, utilizing orthogonal aminoacyl-tRNA synthetase (aaRS)/$tRNA_{CUA}$ pairs, has facilitated the site-specific incorporation of unnatural amino acids into proteins in *Escherichia coli*, yeast, and cultured mammalian cells.[1−6] The application of unnatural amino acid mutagenesis to the production of recombinant proteins allows access to modified proteins, including proteins bearing defined post-translational modifications, for structural biology, enzymology, and single-molecule studies.[6−13] The genetically encoded incorporation of photocaged amino acids in living cells allows the photo-control of protein interactions, protein localization, enzymatic activity, and cellular signaling,[3,14,15] while the incorporation of photo-cross-linking amino acids allows the mapping of weak or transient protein interactions, including those in membranes, that are challenging to define by non-covalent approaches.[16−20] The incorporation of bio-orthogonal chemical handles and biophysical probes is facilitating emerging approaches for protein imaging and spectroscopy.[2,11,21−29]

The genetically encoded site-specific incorporation of unnatural amino acids into proteins in multicellular organisms would facilitate the extension of molecular tools for dissecting and controlling biological processes inside cells[3,14,15,30] to the study of complex processes in whole organisms. This is important because many fundamental biological processes, including those involved in neural processing and development—where unnatural amino acid mutagenesis could provide much needed new insight—can only be studied in animals. Here we report the first genetic code expansion in an animal, the nematode worm *Caenorhabditis elegans*.

C. elegans is an attractive target for a multicellular genetic code expansion. Its genome is sequenced,[31] and the lineage of every cell during embryogenesis and post-embryonic development has been mapped,[32,33] which is invaluable in understanding mutant phenotypes at the cellular level. The organism has around 1000 somatic cells that make up a variety of tissues including muscles, nerves, and intestines. The entire organism is transparent at every stage of life, making it possible to visualize expression in individual cells using fluorescent proteins. This will facilitate light-mediated intervention in biological processes using genetically encoded photo-responsive amino acids, including photo-cross-linkers and photo-caged amino acids, as well as unnatural amino acid-based imaging methods. Many biochemical and signaling pathways involved in disease are conserved between *C. elegans* and humans, which makes *C. elegans* an important organism for identifying the molecular mechanisms of disease.[34] Moreover, *C. elegans* is the only multicellular organism in which amber suppressors have been isolated and introduced into the germ line by classical genetics approaches,[35−38] and suppression efficiencies exceeding 30% have been reported.[39] These observations suggest that amber suppression is not problematic for the organism through its development and reproduction.

The site-specific incorporation of unnatural amino acids into target proteins poses a number of challenges: we require an orthogonal amber suppressor tRNA, that is correctly transcribed, processed, modified, and exported to the cytoplasm of the cell, an orthogonal aaRS that can uniquely aminoacylate the orthogonal tRNA in the cytoplasm, and an mRNA encoding a gene of interest bearing an amber codon that directs amino acid incorporation.[2,40] In addition, we need to combat any effects of nonsense-mediated decay (NMD)[41,42] that may destroy transcripts bearing amber codons and limit expression of proteins bearing unnatural amino acids. The site-specific incorporation of unnatural amino acids in an animal poses additional challenges, since each of the translational components must be present in the same cell or cells within the organism to effect genetic code expansion, and we need to ensure that the unnatural amino acids are taken up by the animal and are available, within the cytoplasm of its cells, for protein translation.

We created a reporter for amber suppression, *Prps-0::mGFP-TAG-mcherry-HA-NLS*, in which a 5′ mGFP is separated from a 3′ mCherry gene by a linker region containing an amber stop codon (Figure 1). A ribosomal protein promoter (*Prps-0*) in this construct drives expression in most cells in the worm,[43] the HA tag allows detection of expression by anti-HA antibodies, the nuclear localization sequence (NLS) concentrates fluorescence in the nucleus, and the *unc-54* 3′ untranslated region stabilizes the mRNA throughout the worm. We injected this reporter into *C. elegans* using a construct carrying wild-type *lin-15B* as a selection marker in a *lin-15B(n765)* genetic background.[44] We observed that the transmission frequency of the transgenic extrachromosomal arrays to offspring was low (20−30%). This resulted in *C. elegans* populations where a majority of animals did not carry the transgenes. Moreover, we observed that the GFP signal in worms carrying the reporter was much weaker than the GFP signal produced from a simple GFP gene.

We reasoned that the low GFP expression was likely due to the degradation of reporter mRNA through NMD.[41,42] When we crossed worms expressing the reporter with *smg-2(e2008)* worms that are deficient in NMD,[41,45] but otherwise healthy,

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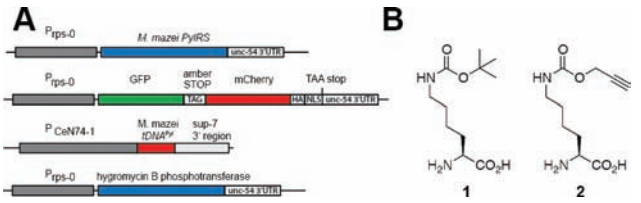

Figure 1. Expanding the genetic code of *C. elegans*. (A) DNA constructs used for genetic code expansion in *C. elegans*. (B) Amino acid substrates for PylRS used in this study.

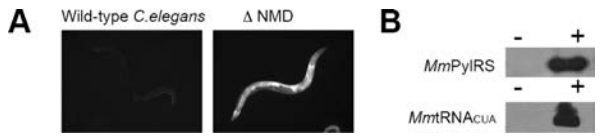

Figure 2. Each of the components required for genetic code expansion in *C. elegans* is expressed in the animal. (A) Effect of nonsense-mediated decay (NMD) on GFP expression levels from worms containing the reporter construct *Prps-0::mGFP-TAG-mCherry-HA-NLS*. "Wild-type *C. elegans*" shows the GFP fluorescence of a representative wild-type animal. "ΔNMD" shows the GFP fluorescence of a transgenic worm created by crossing the reporter construct into the *smg-2(e2008)* mutant background. (B) FLAG-*Mm*PylRS (top) and *Mmt*RNA$_{CUA}$ (bottom) are expressed from animals containing *Prps-0::FLAG-MmPylRS* and *PCeN74-1::MmPylT*. FLAG-*Mm*PylRS was detected by western blot in worm lysates using an anti-FLAG antibody. *Mmt*RNA$_{CUA}$ was detected by northern blot from total RNA isolated from worms. All experiments used a mixed-stage population.

we observed a striking increase of GFP signal (Figure 2 and Supporting Information (SI) Figure 1). While we see a strong GFP signal in worms transformed with the reporter, we do not observe any mCherry fluorescence, demonstrating that the reporter is functional and that the worms do not contain endogenous amber suppressors. We constructed all subsequent transgenic lines using the *smg-2(e2008)* worms.

To address the problem of low transmission levels, we tested transformation markers that use a gene conferring resistance to specific antibiotics. Recent reports use puromycin[46] or G-418[47] resistance genes for antibiotic-based selection in worms. However, puromycin efficiently kills wild-type animals only in the presence of the permeabilizing detergent, Triton X-100, and G-418 does not kill all wild-type worms in a population. We therefore investigated a further antibiotic, hygromycin B,[48] which has not been used as a selectable marker in *C. elegans*. We found that hygromycin B (0.5 mg/mL) kills 100% of wild-type worms without the addition of Triton X-100 (data not shown). When the hygromycin B phosphotransferase gene (*hpt*) fused to the *rps-0* promoter (*Prps-0::hpt*) was injected into worms, it conferred resistance to the antibiotic. Using the *hpt* transformation marker, we were able to isolate transgenic lines that appear to have transmission rates of 100% in the presence of hygromycin B (data not shown). In all subsequent experiments, we used hygromycin B resistance to maintain DNA constructs into *C. elegans*.

Three aminoacyl-tRNA synthetase/tRNA$_{CUA}$ pairs (*Ec* tyrosyl-tRNA synthetase/tRNA$_{CUA}$, *Ec* leucyl-tRNA synthetase/tRNA$_{CUA}$, and pyrrolysyl-tRNA synthetase (PylRS)/tRNA$_{CUA}$ from *Methanosarcina* species) are orthogonal in eukaryotic cells and have been used to incorporate unnatural amino acids.[2,3,5,14,49,50] We and others have demonstrated that the PylRS/tRNA$_{CUA}$ pairs from *Methanosarcina* species including *M. barkeri* (*Mb*) and *M. mazei* (*Mm*), which naturally uses pyrrolysine, can be used to incorporate a range of unnatural amino acids, including *N*ε-(*tert*-butyloxycarbonyl)-L-lysine (**1**) and *N*6-[(2-propynyloxy)carbonyl]-L-lysine (**2**).[3−6,11,26,27,51−54] The PylRS/tRNA$_{CUA}$ pair, unlike the other pairs that are orthogonal in eukaryotes, can be rapidly evolved in *E. coli* to recognize new amino acids, and numerous unnatural amino acids can now be incorporated using this pair and its evolved variants. Moreover, PylRS variants evolved in *E. coli* can be transplanted into eukaryotic cells,[3,5,14,50,55] making it especially attractive to develop this pair for incorporating unnatural amino acids in animals.

To express *Mm*PylRS from an RNA Polymerase II (Pol II) promoter, we created *Prps-0::FLAG-MmPylRS,* in which *Prps-0* directs expression throughout the animal. Western blots demonstrate that the synthetase is expressed in worms (Figure 2 and SI Figure 1).

*Mmt*RNA$_{CUA}$ requires RNA polymerase III transcription. Transcription of eukaryotic tRNAs by RNAP III is directed by A and B box sequences that are internal to the tRNA gene. These sequences are not present in the orthogonal *Mmt*RNA$_{CUA}$ gene, and it is challenging to introduce them without disrupting tRNA function.[4] We therefore investigated extragenic RNA polymerase III promoters for the transcription of *Mmt*RNA$_{CUA}$. To direct the transcription of *Mmt*RNA$_{CUA}$, we created *PCeN74-1::MmPylT::sup-7* 3′, in which the selected Pol III promoter, derived from the stem-bulge non-coding RNA CeN74-1, is fused to the 5′ end of the *Mmt*RNA$_{CUA}$ gene and transcription of the tRNA is terminated by the region found immediately 3′ of the *sup-7 C. elegans* tryptophanyl tRNA gene. We chose the CeN74-1 promoter, since it shows a high level of expression in adult animals and some expression in larval stages;[56,57] we reasoned that these properties would enable us to more efficiently screen for cells or animals expressing a functional tRNA, since worms are in the adult stage for up to several weeks but are only in the larval stages for a short period. Northern blots, using a probe specific for *Mmt*RNA$_{CUA}$,[4] demonstrate that the tRNA is efficiently produced from this promoter in *C. elegans* (Figure 2 and SI Figure 1).

We constructed lines containing all genetic components by biolistic bombardment[58] of *smg-2(e2008)* worms with plasmids encoding the reporter, synthetase, tRNA and hygromycin B phosphotransferase gene (*Prps-0::mGFP-TAG-mcherry-HA-NLS, Prps-0::FLAG-MmPylRS, PCeN74-1::MmPylT, Prps-0::hpt*). The transformants were grown on plates supplemented with hygromycin B for 2 weeks to kill off all non-transgenic worms, resulting in populations where all worms contained the extrachromosomal transgenic array *Ex1* [*Prps-0::mGFP-TAG-mcherry-HA-NLS, Prps-0::FLAG-MmPylRS, PCeN74-1::MmPylT, Prps-0::hpt*]. Surviving worms were grown on 5 mM **1** and inspected by fluorescence microscopy for the presence of mCherry in the nucleus of cells within the worm. This step allowed us to select for animals expressing the reporter as well as functional *Mm*PylRS and *Mmt*RNA$_{CUA}$.

We examined several thousand worms and observed a few (1−5) mCherry-positive worms per hundred worms examined. Individual worms showed mCherry expression in different tissues, including intestinal cells, pharyngeal cells, neurons, and body wall muscle. The mosaicism of expression from these extrachromosomal arrays is well documented and may result from either loss of the array during mitosis or partial or complete silencing of the array.

We singled out 13 mCherry positive worms and grew them in the absence of **1** and the presence of hygromycin B, to select for inheritance of the array in the resulting lines. We examined these lines for mCherry fluorescence in the presence and in the absence of **1**. While all lines selected showed amino acid-dependent mCherry fluorescence, we focused in subsequent experiments on two lines (1.3.1 and 1.8.1). These lines were singled out from distinct plates and showed the strongest mCherry fluorescence in the presence of amino acid **1**. In the absence of amino acid **1**, we did not find any worms expressing mCherry in the several thousand animals we screened by fluorescence microscopy. In contrast, when amino acid **1** was added to the lines, we saw strong mCherry fluorescence that was easily detectable by eye under a dissection microscope (Figure 3A and SI Movies 1−4) in a fraction of the worms (~5%). The mCherry fluorescence was nuclear, consistent with the mCherry-GFP fusion bearing a nuclear localization sequence. In contrast, GFP fluorescence was diffuse, as free GFP resulting from termination at the amber codon is found throughout the cell. Between animals in a single line, we observed variation in both the number and identity of cells displaying mCherry fluorescence. This may result from loss of the extra-chromosomal array during developmental mitosis and/or partial silencing of the extra-chromosomal array, leading to silencing of at least one essential genetic component (synthetase or tRNA or reporter).

To further demonstrate that the unnatural amino acid is incorporated in response to the amber codon, we lysed worms from each line grown in the presence and in the absence of **1** for western blotting. Anti-HA and anti-GFP western blots confirmed the unnatural amino acid-dependent production of GFP-mCherry-HA-NLS in worms (Figure 3B and SI Figure 2).

To demonstrate the generality of our approach for incorporating unnatural amino acids using the *Mm*PylRS/*Mm*tRNA$_{CUA}$ pair in *C. elegans*, we performed experiments using **2**, that we have previously site-specifically incorporated into proteins in *E. coli* and eukaryotic cells using PylRS.[4,27] Worms from line 1.8.1 displayed mCherry fluorescence in the presence, but not in the absence, of **2** (SI Figure 3). When an anti-mCherry antibody was used to immunoprecipitate proteins from line 1.8.1, the production of full-length protein, as detected with an antibody against the C-terminal HA tag, was dependent on the presence of either **1** or **2**. GFP-mCherry-HA-NLS purified from worms grown in the presence of **2** was selectively labeled with biotin-azide **3** (SI Figure 3B), via a copper(I) catalyzed cycloaddition,[59] confirming the incorporation of the alkyne (Figure 3C).

Taken together, the fluorescence imaging and western blot data demonstrate that the *Mm*PylRS/*Mm*tRNA$_{CUA}$ pair directs the incorporation of unnatural amino acids, **1** or **2**, in response to an amber stop codon in *C. elegans*.[60] The incorporation of **2** in *C. elegans* was directly confirmed by its specific and selective labeling with the biotin azide, **3**.

In the absence of added unnatural amino acid, no full-length protein is produced, demonstrating that *Mm*tRNA$_{CUA}$ is not appreciably aminoacylated by endogenous synthetases and is orthogonal in worms. The orthogonality of *Mm*PylRS in *C. elegans* is confirmed by experiments demonstrating that the *Mm*PylRS/*Mm*tRNA$_{CUA}$ does not appreciably incorporate **2** in response to sense codons[61] (SI Figure 4).

In conclusion, we have demonstrated the first genetically encoded incorporation of unnatural amino acids in a multicellular organism. Since we see mCherry expression throughout the organism, our data suggest that the *Mm*PylRS/*Mm*tRNA$_{CUA}$ pair can function in diverse tissues to incorporate unnatural amino acids. The PylRS/tRNA$_{CUA}$ pairs and their derivatives that have been evolved in *E. coli* can be used to direct the incorporation of a range of unnatural amino acids; extensions of the approach reported here should allow the introduction of post-translational modifications, photocaged amino acids, bioorthgonal chemical handles, and photo-cross-linkers into proteins in *C. elegans*. The approach we have developed may provide tools to dissect the molecular basis of complex biological phenomena in whole animals.

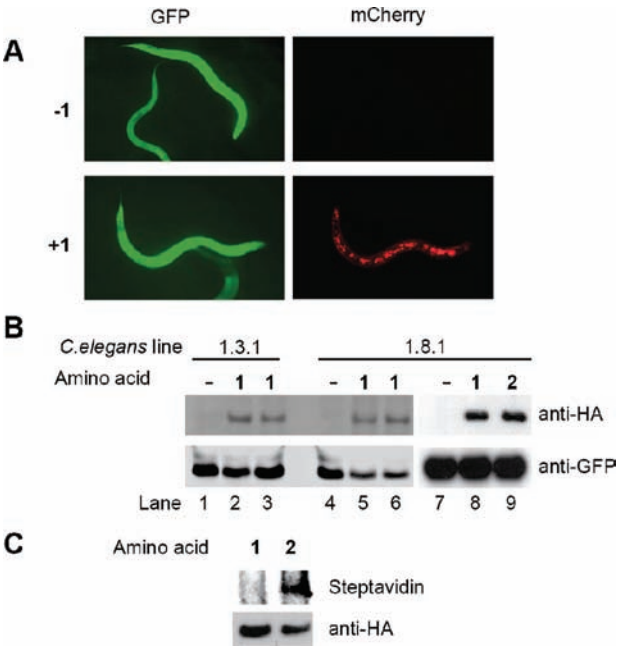

Figure 3. The orthogonal *Mm*PylRS/*Mm*tRNA$_{CUA}$ pair incorporates **1** or **2** in response to the amber codon in *C. elegans*. (A) Fluorescence images of worms containing *Ex1* [*Prps-0::mGFP-TAG-mcherry-HA-NLS, Prps-0::FLAG-MmPylRS, PCeN74-1::MmPylT, Prps-0::hpt*] in the absence (top) and in the presence (bottom) of **1** (see also SI Movies 1−4). Both GFP and the GFP::mCherry fusion protein show mosaic expression throughout the animal. GFP mosaicism is not visible here because its fluorescence is saturating under the imaging conditions, but GFP mosaicism is clear in Figure 2A. mCherry is nuclear localized, making its mosaicism more obvious. (B) Lanes 1−6: western blot of lysates of worms grown in the absence or presence of **1**. Lanes 7−9: western blot of GFP::mCherry fusion protein affinity purified using an antibody against mCherry. GFP::mCherry was detected using an antibody against the C-terminal HA tag. Anti-GFP blots are loading controls (lanes 1−6) and input controls (lanes 7 and 8). More protein was loaded in the "no added amino acid" lanes (lanes 1 and 4). (C) Genetically encoded **2** can be specifically labeled with an azido derivative of biotin, **3**. Immunoprecipitated GFP::mCherry fusion from B was labeled with **3** and the reaction products were detected using streptavidin.

■ ASSOCIATED CONTENT

Supporting Information. Complete refs 43 and 56, methods, supplementary figures, and supplementary movies. This material is available free of charge via the Internet at http://pubs.acs.org.

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(60) While we have demonstrated that **1** is incorporated in an unmodified form in bacteria and eukaryotic cells,[4,27] we cannot currently rule out that a non-conserved activity in worms may post-translationally modify **1**.
(61) The orthogonality of the synthetase and tRNA is best addressed in a single line of worms, rather than in separate lines bearing only the synthetase or tRNA, which may have different genetic backgrounds.