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Protein & Cell

LETTER

Light-induced protein translocation by genetically encoded unnatural amino acid in *Caenorhabditis elegans*

Dear Editor,

Living systems such as *Caenorhabditis elegans* are organized by interaction networks of biopolymer and small molecules. The molecular engineering of specific targets to monitor and control these biological molecules has led to significant advances in our understanding of how biological systems are organized, maintain themselves and disintegrate (Prescher and Bertozzi, 2005). For instance, green fluorescent protein (GFP) derived from jellyfish *Aequorea Victoria* helps us to accurately locate and observe proteins over long term periods. In addition, many novel LOV (light, oxygen, or voltage) domains such as LOV2-Jα and Vivid offer the possibility of cell-based motility management and tissue-based gene expression control (Wu et al., 2009; Wang et al., 2012). However, conjugated targets may lead to artificial localization of proteins in particular membrane biopolymers (Zhang et al., 2012). One technique to solve this problem is utilizing unnatural amino acids (Uaas) as labeling systems (Wang et al., 2001, 2006, 2009). In order to incorporate unnatural amino acids into specific sites of a protein, three elements are essential: an orthogonal tRNA-codon pair, a corresponding amino acyl-tRNA synthetase and a synthetic unnatural amino acid (Wang et al., 2006). By expanding the canonical genetic code with Uaas, new properties can be integrated into proteins, which were demonstrated to be useful for the study of many biological phenomena such as analysis of protein structure, regulation of metabolism activity, monitoring of proteins and many others (Wang et al., 2009).

During the past ten years, the Uaa-labeling method has been successfully expanded to researches of *Escherichia coli*, yeast and mammalian cell systems. In 2011, for the first time Uaas were incorporated into multicellular organisms the nematode worm *Caenorhabditis elegans* (Greiss and Chin, 2011). In this research, worms with a *smg-2*(*e2008*) background were used to increase the incorporating efficiency. Since *smg-2* regulates the accumulation of nonsense mRNA and bursa swollen, it may also lead to artificial results for functional analysis. In addition, the antibiotic drug was used as a selection tool against unlabelled worms. Moreover, the efficiency of Uaas incorporation is very low.

In this paper, we reported a new strategy to incorporate photo-caged lysine into wild type *C. elegans* with harmless protocols and a very high successful rate. First, we screened different promoters for efficient tRNA expression and found that a type-3 Poly III promoter *U6-10* could express orthogonal tRNA-codon pair with high efficiency. Next, we utilized a liquid culture approach instead of the palette medium culturing method. The incorporation efficiency of Uaas was more than 50%. With this method, we successfully incorporated modified *o*-nitrobenzyl (ONB)-lysine (ONBK) into GFP-tagged nuclear localization signal peptide. After 405 nm laser illumination, photo-caged lysine was converted to lysine and directed NLS-GFP to re-localize to nucleus in worm muscle and intestine cells. In conclusion, we have demonstrated that *C. elegans* is accessible for incorporation of Uaas as a powerful tool for studying intracellular processes and the distribution of target protein can be controlled by non-invasive light modulation.

To achieve the site-specific incorporation of ONBK into worm proteins, it is necessary to express orthogonal aminoacyl-tRNA synthetase, the ONBK-PylRS, and pyrrolysine $tRNA_{CUA}$ ($tRNA^{Pyl}_{CUA}$) in the same cells. To express *PylT*, the gene of $tRNA^{Pyl}_{CUA}$, type-3 Poly III promoters which were reported previously to be the most efficient promoters were the best choices (Wang et al., 2007). Since there were no suitable worm type-3 Poly III promoters reported which could express orthogonal $tRNA^{Pyl}_{CUA}$ efficiently, 19 type-3 Poly III promoters including eighteen *U6* promoters and 1 *RNA P RNA* (*H1*) promoter were selected as candidates. First, we separated them into five groups using MEME software based on three conserved motifs (PSEA, PSEB and [G]TATA box) (Fig. S1) (Li et al., 2008; Bailey et al., 2009).

Next, five representative promoters from different groups named *U6-2*, *4*, *10*, *12* and *H1* were selected and cloned upstream of $tRNA^{Pyl}_{CUA}$ sequence. The 3′ flanking sequence of *C. elegans* endogenous amber suppressor *sup-7* was used as a stopping and processing signal of the orthogonal tRNA. Type-3 Poly III promoters cannot drive the translation of reporter genes such as GFP or mCherry. However, if a trans-splicing element such as SL2 is inserted before the reporter gene, the reporter gene would be translated into proteins successfully. Thus, the sequence of *SL2*::*mCherry* was added downstream the tRNA sequence and utilized to report the expression pattern of $tRNA^{Pyl}_{CUA}$

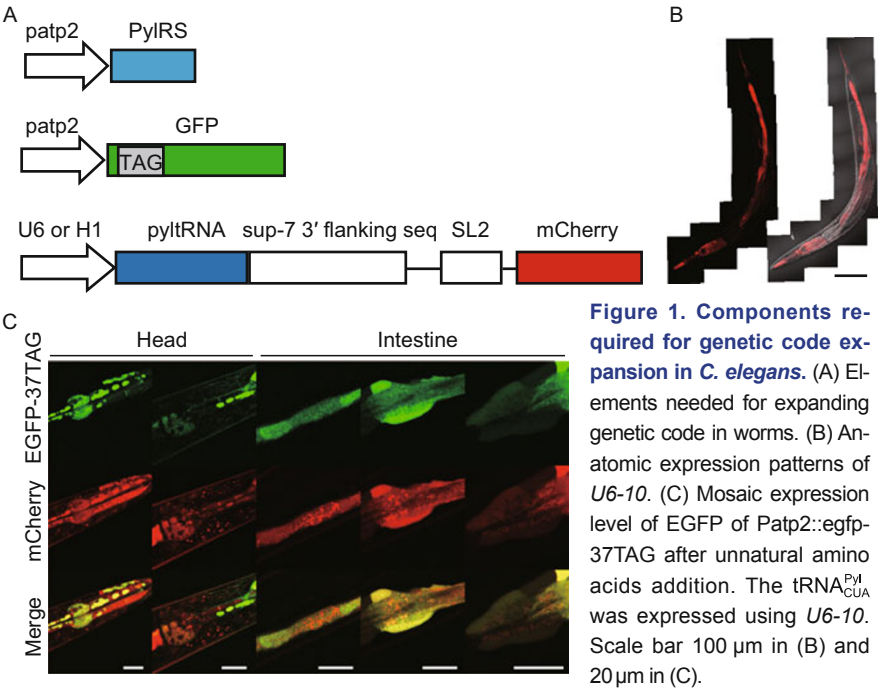

Figure 1. Components required for genetic code expansion in *C. elegans*. (A) Elements needed for expanding genetic code in worms. (B) Anatomic expression patterns of *U6-10*. (C) Mosaic expression level of EGFP of Patp2::egfp-37TAG after unnatural amino acids addition. The $tRNA^{Pyl}_{CUA}$ was expressed using *U6-10*. Scale bar 100 μm in (B) and 20 μm in (C).

(Fig. 1A). Using mCherry as a reporter, we first evaluated the expression pattern of *U6-2*, *4*, *10*, *12* and *H1* and found nearly all of them except *U6-4* could drive efficiently expression in various tissues including intestine, muscle and pharynx (Fig. 1B).

The $tRNA^{Pyl}_{CUA}$ expression level was then evaluated and measured by Northern blotting. Different stable strains of transgenic worms containing the individual promoter constructs of *U6-2*, *10*, *12* and *H1* were established and enlarged for total RNA isolation and purification. Very low signal was detected for the *U6-12* promoter construct. By contrast, the *U6-2* and *U6-10* constructs expressed $tRNA^{Pyl}_{CUA}$ at very high level, with ~9-fold and ~15-fold compared to *U6-12*, respectively (Fig. S2).

To test whether promoters *U6-2* and *U6-10* could generate functional amber suppressor $tRNA^{Pyl}_{CUA}$ in worms, the constructs containing the promoter *apt-2* and downstream ONBK-PylRS and EGFP-37TAG (a TAG mutant at the 37th site of EGFP) were co-injected into *C. elegans* with either $P_{U6\text{-}2}$::*PylT or* $P_{U6\text{-}10}$::*PylT*. Transgenic young adult worms from stable lines were selected and cultured for 4 days in S-basal solution containing 5 mmol/L ONBK (Fig. S3A) and concentrated OP50 bacteria. In the F1 generation, there were substantial numbers of worms expressing bright green fluorescence assessed by fluorescent confocal microscopy (Fig. 1C), which was also proved by Western blotting using GFP antibody (Fig. S4). As a control, under the same condition without ONBK, no EGFP signal was detected (Fig. S5). The results indicated that functional ONBK-conjuncted aminoacyl-tRNA were indeed biosynthesized. As shown in Fig. 1C, the EGFP signal and the mCherry signal matched well, indicating that functional aminoacyl-tRNA is required for the read through of amber stop codon in EGFP-37TAG.

We next checked the incorporation efficiency by measuring the number of Uaa-labelled fluorescent worms in offspring. Initially, ONBK was mixed with OP50 bacteria and seeded on Nematode Growth Medium (NGM) plates. Less than 1% of total offspring could be detected with EGFP signal. Unlike cultured mammalian cells, it is difficult to deliver Uaas into the cells of multicellular animals. Worm has a protective cuticle that blocks efficient absorption of many compounds and intestinal transporter for natural amino acids may not recognize the modified Uaas. To increase the incorporation efficiency of Uaas, we tried liquid culture, instead of NGM plates, and found that more than 50% of offspring with red fluorescence signal showed green fluorescence for both *U6-2* and *U6-10* promoter constructs, as estimated by Complex Object Parametric Analysis and Sorter (COPAS) (Han et al., 2013) (Fig. S6). However, in the stable *U6-2* line, ~8% of offspring could also exhibit EGFP fluorescence in the absence of ONBK incubation, indicating that this line has background readthrough for some reasons (Fig. S6C). Thus, the promoter *U6-10* appeared to be a better choice for the expression of functional tRNA, whilst also limiting levels of background fluorescence.

ONBK is an unstable lysine derivative, and its ONB group is readily removed upon UV light exposure (Chen et al., 2009; Gautier et al., 2010). To test the functional consequence of ONBK incorporation, we employed two constructs as previously reported: 1) PCKRS (a PylRS mutant)/tRNA pair driven under the promoters of *atp-2* and *U6-10*, respectively; 2) a nuclear localization signal with a lysine mutated to amber stop codon (shorted for NLS^{TAG}) conjuncted with TagGFP (a homologue of GFP) driven by the promoter *atp-2* (Fig. 2A). In addition, we used a modified ONBK to increase the decaging efficiency which was reported previously (Fig. S3B) (Gautier et al., 2010). When modified ONBK was added into liquid cultured stable transgenic strain, NLS^{TAG}::TagGFP was successfully read through and expressed. GFP signals were both detected in the nucleus as well as in the cytoplasm of pharyngeal muscle and intestinal cells, in consistent with previous results obtained in mammalian cells (Fig. 2B). The worm was illuminated under 405 nm light for 30 s, and by confocal microscope we found that a significant increase of green fluorescent in nucleus with a concomitant decrease of fluorescence in cytoplasm (Fig. 2B and 2C). We plotted the intensity of fluorescence in nucleus and cytoplasm respectively after 405 nm exposure in 9 individual cells of different worm (Fig. 2D), and calculated the ratio

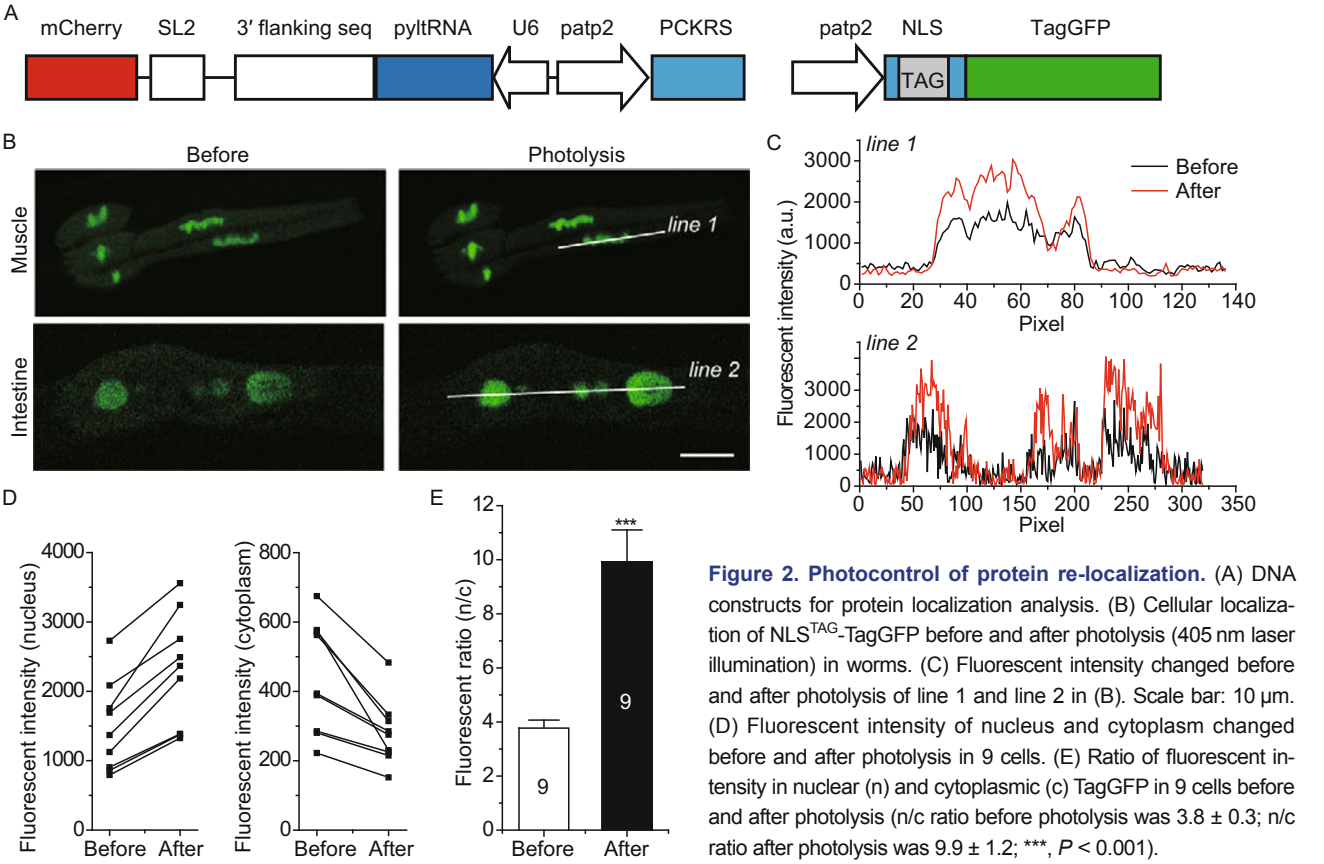

Figure 2. Photocontrol of protein re-localization. (A) DNA constructs for protein localization analysis. (B) Cellular localization of NLS^{TAG}-TagGFP before and after photolysis (405 nm laser illumination) in worms. (C) Fluorescent intensity changed before and after photolysis of line 1 and line 2 in (B). Scale bar: 10 μm. (D) Fluorescent intensity of nucleus and cytoplasm changed before and after photolysis in 9 cells. (E) Ratio of fluorescent intensity in nuclear (n) and cytoplasmic (c) TagGFP in 9 cells before and after photolysis (n/c ratio before photolysis was 3.8 ± 0.3; n/c ratio after photolysis was 9.9 ± 1.2; ***, $P < 0.001$).

of fluorescent intensity of the nucleus to the cytosolic before and after the light exposure (Fig. 2E). The results show that photolysis of photo-caged lysine directed NLS-GFP to re-localize to nucleus in worm muscle and intestine cells.

In summary, we have set up a method to expand the genetic code of *C. elegans* by introducing a PylRS and $tRNA^{Pyl}_{CUA}$ pair. Our strategy contained two important elements. First, we screened for the strongest native type-3 Poly III promoter to drive the expression of tRNA and found one of the type-3 Poly III promoters, *U6-10*, gave the strongest expression of functional pyrrolysine tRNA without detectable background read-through in the absence of unnatural amino acids. Second, using liquid culture instead of solid NGM plates could largely increase the incorporation efficiency of unnatural amino acids into worms. Worms cultured in liquid media are kept in an a priori state of calorie restriction, which has been shown to alter multiple pathways of energy metabolism (Castelein et al., 2008). It is possible that worms under diet restriction will take up more unnatural amino acids. Using this method, an unstable unnatural amino acid, the modified ONB-lysine, was successfully incorporated into worms for the first time. We also proved that the active group of modified ONB-lysine would retain for a long time in liquid culture. This approach should provide a powerful tool to study the function of proteins *in vivo* by acutely activating or deactivating proteins using light, which is clearly superior to chronic knockout of proteins. The spatiotemporal control function of a protein by photolysis of a caged-lysine or other caged-Uaas makes it possible to check immediate behaviours of worms, like mechanosensation, chemosensation and locomotion, through the gain-of-function of the protein by photolysis.

FOOTNOTES

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Hao Chang, Mei Han, Wenming Huang, Guifeng Wei, Juanjuan Chen, Peng R. Chen, Runsheng Chen, Junlong Zhang, Tao Xu and Pingyong Xu declare that they have no conflict of interest. All institutional and national guidelines for the care and use of laboratory animals were followed.

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