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Expanding the genetic code of *Drosophila melanogaster*

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Genetic code expansion for unnatural amino acid mutagenesis has, until recently, been limited to cell culture. We demonstrate the site-specific incorporation of unnatural amino acids into proteins in *Drosophila melanogaster* at different developmental stages, in specific tissues and in a subset of cells within a tissue. This approach provides a foundation for probing and controlling processes in this established metazoan model organism with a new level of molecular precision.

Genetic code expansion, using orthogonal amber suppressor aminoacyl-tRNA synthetase/$tRNA_{CUA}$ pairs[1,2], has facilitated the site-specific incorporation of unnatural amino acids into proteins in *Escherichia coli*, yeast, cultured animal cells and, most recently, *Caenorhabditis elegans*[1,2]; this has allowed new insights into the structure and function of proteins, their complexes and their post-translational regulation[1]. The incorporation of photoresponsive amino acids into proteins in living cells has allowed the control of enzymatic activity, molecular interactions and protein transport with light and has provided insights into the kinetics of signaling[3–5], whereas the incorporation of photocrosslinking amino acids has allowed the mapping of weak and transient protein interactions in cells[1]. Addressing the challenges of achieving genetic code expansion in whole animals will allow the extension of these strategies to the study of processes, including embryonic development, tissue morphogenesis, tumor biology and neuronal plasticity, at the organism level.

The fruit fly *D. melanogaster* is perhaps the most established metazoan model organism[6]. It has a short generation time and an extensively characterized and sequenced genome, and there are established tools for creating transgenic flies and controlling transgene expression[7–9]. The early *D. melanogaster* embryo is ideally suited to the use of photoresponsive amino acids or amino acids for imaging applications, and amber suppressor tRNAs have been generated by mutation of *D. melanogaster* tRNAs, leading to amber suppression in the fly[10–12].

The pyrrolysyl-tRNA synthetase (PylRS)/$tRNA_{CUA}$ pairs from *Methanosarcina* species including *M. barkeri* and *M. mazei* can be used to incorporate a range of unnatural amino acids, including Nε-(tert-butyloxycarbonyl)-L-lysine (**1**), Nε-5-norbornene-2-yloxy-carbonyl-L-lysine (**2**) and *N*6-[(2-propynyloxy)carbonyl]-L-lysine (**3**)[13–15] (**Fig. 1a**). The PylRS/$tRNA_{CUA}$ pair has been rapidly evolved in *E. coli* to recognize a wide range of useful new amino acids[16,17]. PylRS variants evolved in *E. coli* can be transplanted into eukaryotic cells[4,18,19], making it especially attractive to develop this pair for incorporating unnatural amino acids in animals[20]. Here we demonstrate the site-specific incorporation of unnatural amino acids in *D. melanogaster* (**Supplementary Results**, **Supplementary Fig. 1**).

We cloned *M. mazei* PylRS under the transcriptional control of an upstream activating sequence (UAS) recognized by GAL4 (ref. 8) (**Supplementary Methods**, **Fig. 1b**). We expressed *M. mazei* $tRNA_{CUA}$ (*Mm*$tRNA_{CUA}$), encoded by *PylT* (AE008384.1; 1729220 to 1729280) using an extragenic Pol III promoter that normally drives expression of U6 spliceosomal RNA[21], a strategy that has been successful for expressing *PylT* in eukaryotic cells[4,20,22]. To report on

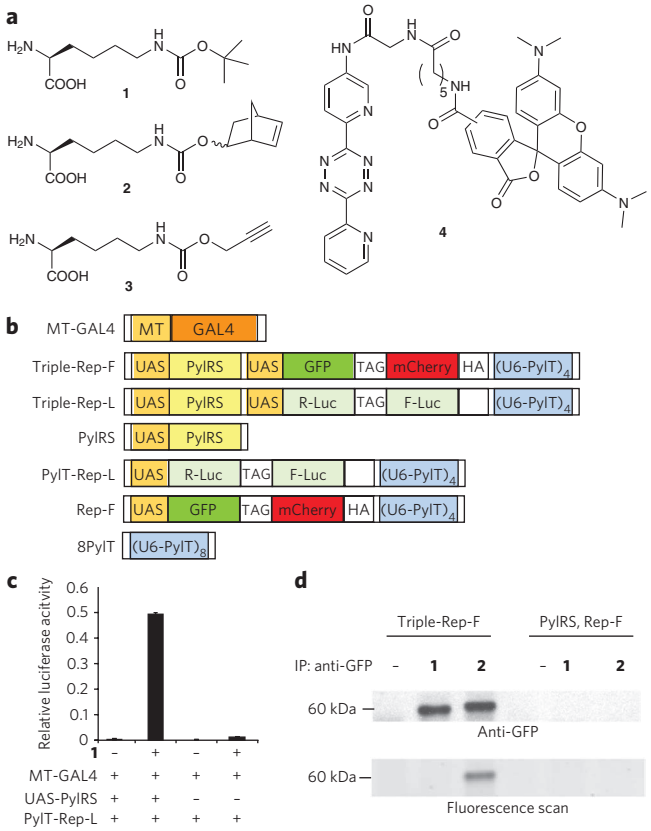

Figure 1 | Site-specific incorporation and labeling of unnatural amino acids in *D. melanogaster* Dmel cells using an orthogonal *Mm*PylRS/$tRNA_{CUA}$ pair. (**a**) Unnatural amino acid and tetrazine probes used in this study. (**b**) Constructs used in the present study. MT, metallothionein promoter; GAL4, a yeast transcription factor; HA, hemagglutinin tag; R-Luc, *Renilla* luciferase; F-Luc, firefly luciferase; UAS, a GAL4-responsive upstream activating sequence; TAG, amber stop codon; U6-PylT, the U6 promoter followed by a copy of the *PylT* gene encoding *Mm*$tRNA_{CUA}$. (**c**) Relative luciferase activity detected in cell extracts from transfected Dmel cells. The data show the mean of three trials ± s.d. (**d**) Immunoprecipitation (IP) with anti-GFP from Dmel cells transfected with the indicated constructs and MT-GAL4 and incubated in the presence of the indicated amino acid. The fluorescence scan detects labeling with **4**. The full gel is in **Supplementary Figure 4**.

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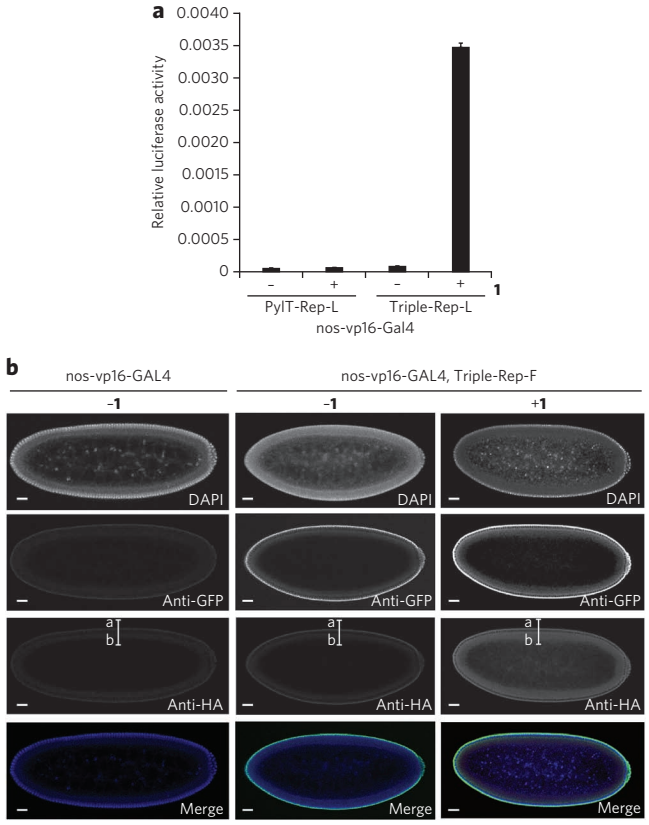

Figure 2 | Site-specific incorporation of unnatural amino acids in *D. melanogaster* embryos. (**a**) Dual luciferase activity detected in embryonic extracts of the indicated genotype. The data show the mean of three trials ± s.d. (**b**) Embryos of the indicated genotype were stained as indicated. Scale bars, 20 μm. The a-b line indicated was used for quantification in **Supplementary Figure 7**.

unnatural amino acid incorporation, we created two independent reporter systems (**Fig. 1b**): a dual luciferase reporter and a fluorescent protein fusion reporter, each under control of a UAS.

To demonstrate that the *Mm*PylRS/tRNA$_{CUA}$ pair is expressed, orthogonal to endogenous synthetases and tRNAs, and functions with the *D. melanogaster* translational machinery to direct the incorporation of unnatural amino acids in response to the amber codon, we performed experiments in a *D. melanogaster* cell line (Dmel).

Cells were transfected with plasmids encoding GAL4 under the control of a metallothionein promoter (MT-GAL4)[23], PylRS and PylT-Rep-L (**Fig. 1b**). We induced GAL4 expression by the addition of Cu^{2+}. In the presence of amino acid **1** (10 mM; a substrate for the PylRS/tRNA$_{CUA}$ pair)[1], we observed a dual luciferase signal that was at least 50-fold greater than that observed in control experiments in which amino acid **1** or PylRS was omitted (**Fig. 1c**). We observed the same amino acid dependence with Triple-Rep-L (**Fig. 1b**) and demonstrated that amino acids **1**, **2** and **3** can be incorporated (**Supplementary Fig. 2**).

To provide independent confirmation of both the unnatural amino acid incorporation observed with the *Mm*PylRS/tRNA$_{CUA}$ pair and the orthogonality of *Mm*tRNA$_{CUA}$, we used a fluorescent protein fusion reporter. Dmel cells were transfected with MT-GAL4 and Triple-Rep-F (**Fig. 1b**), and GAL4 expression was induced by the addition of Cu^{2+}. We observed GFP expression in both the presence and absence of **1**, **2** and **3**. However, mCherry fluorescence was observed only in the presence of the unnatural amino acids (**Supplementary Fig. 3**). Western blotting using an antibody against the C-terminal hemagglutinin (HA) tag of the reporter (anti-HA) or against GFP (anti-GFP) detected the 60-kDa GFP-mCherry-HA fusion protein in the presence, but not absence, of the unnatural amino acid **1** (**Fig. 1d** and **Supplementary Figs. 3** and **4**). Similarly, we detected full-length GFP-mCherry-HA protein in the presence but not the absence of **2**, which we have previously site-specifically incorporated into proteins in *E. coli* and mammalian cells using the *Mm*PylRS/tRNA$_{CUA}$ pair[13] (**Fig. 1d** and **Supplementary Fig. 3**). GFP-**2**-mCherry-HA was specifically labeled with a tetrazine fluorophore **4** in a bio-orthogonal inverse electron demand Diels-Alder reaction (**Fig. 1d** and **Supplementary Fig. 5a**)[13]. Control experiments demonstrate that GFP-**1**-mCherry-HA is not labeled with **4**, confirming the specificity of the bio-orthogonal labeling reaction for amino acid **2**. Additional labeling experiments (**Supplementary Fig. 5a–c**) confirmed that *Mm*PylRS does not appreciably direct the incorporation of **2** in response to sense codons. MS confirmed the incorporation of the unnatural amino acids **1**, **2** and **3** at the genetically encoded site (**Supplementary Fig. 6**) and provided further evidence that the unnatural amino acids are not incorporated at sense codons. The data demonstrate that the *Mm*PylRS/tRNA$_{CUA}$ pair is orthogonal to the synthetases and tRNAs in flies and that the pair functions with the fly translational machinery to direct the incorporation of the unnatural amino acids at a genetically encoded site.

We next generated multiple transgenic lines harboring *Mm*PylRS/tRNA$_{CUA}$ and either Triple-Rep-L or Triple-Rep-F (**Fig. 1b**) by P element insertion. As PylRS and the reporter gene are under the control of an UAS, they will not be expressed efficiently in these lines[8,9]. To produce a functional system for the incorporation of unnatural amino acids into proteins in flies, we crossed transgenic flies bearing Triple-Rep-L, PylT-Rep-L or Triple-Rep-F with flies bearing a female germline-specific GAL4 driver, nos-vp16-GAL4 (ref. 24). The resulting transgenic flies were fed for 48 h with or without amino acid **1**, and their embryos were collected over 6 h. The dual luciferase signal from embryos (**Fig. 2a**) was dependent on both the presence of the synthetase and the addition of the unnatural amino acid **1**. We detected GFP-mCherry-HA by immunostaining and western blotting (**Fig. 2b** and **Supplementary Fig. 7**) in transgenic embryos bearing Triple-Rep-F and nos-vp16-GAL4 whose mothers were fed unnatural amino acids (including **3**, a known substrate for PylRS[14]; **Supplementary Fig. 7d**). These data demonstrated the incorporation of **1** and **3** in response to the amber codon in embryos. Unnatural amino acid incorporation in embryos increased as a function of amino acid concentration and synthetase or tRNA gene copy number (**Supplementary Fig. 8a**,**b**).

Dual luciferase activity measured in ovaries, dissected from Triple-Rep-L/nos-vp16-GAL4 (an ovary-specific driver in adult flies) or PylT-Rep-L/nos-vp16-GAL4 flies, was dependent on the addition of amino acid **1** and PylRS (**Supplementary Fig. 8c**). To demonstrate the tissue-specific incorporation of unnatural amino acids, we dissected fly ovaries from the remnants of the fly body in Triple-Rep-F/nos-vp16-GAL4 flies (**Fig. 3a**). Although we detected free GFP in the ovary samples with and without amino acid, we only detected full-length GFP-mCherry-HA in ovaries when flies were fed **1**. We detected neither GFP nor GFP-mCherry-HA in the fly body. Taken together, these data demonstrate that the incorporation of the unnatural amino acid at a specific site in a protein expressed in a specific tissue of interest can be achieved using specific GAL4 drivers. Using an actin-GAL4 driver (Act5CGAL4), we demonstrated that **1** can be incorporated in the fly body and established that feeding flies **1** has no measurable effect on hatching rate or the fraction of Act5CGAL4/Triple-Rep-F flies produced from heterozygous parents, suggesting that unnatural amino acid incorporation has little effect on viability (**Supplementary Fig. 9**). Feeding flies unnatural amino acids for a whole life cycle only

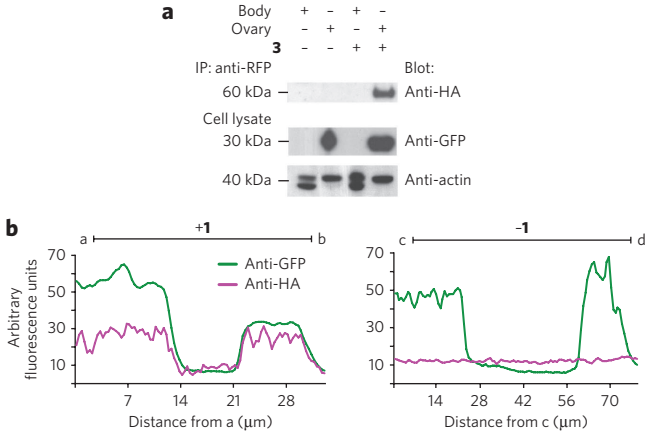

Figure 3 | Tissue-specific and subtissue-specific incorporation of unnatural amino acids in *D. melanogaster* ovaries. (**a**) Top, anti-red fluorescent protein (anti-RFP) immunoprecipitation from nos-vp16-GAL4/Triple-Rep-F extracts from dissected ovaries or the rest of the female body. Bottom, detecting actin and GFP in lysate, different actin variants are detected in the body and ovary. Full gels are in **Supplementary Figure 4**. (**b**) Amino acid **1** is specifically incorporated in mosaic clones generated in the ovary follicular epithelium using an actin-flipout-GAL4 strategy in Triple-Rep-F females. Ovaries were stained with anti-GFP and anti-HA, and cross-section images were obtained by confocal microscopy. The anti-HA and anti-GFP signals were quantified along lines that traverse the boundary between GFP-positive and GFP-negative clones (**Supplementary Fig. 10**). In the presence of the amino acid, there is a statistically significant correlation between the anti-HA and anti-GFP signal ($P = 8.12 \times 10^{-29}$), whereas in the absence of the amino acid there is no significant correlation ($P = 0.36$; Pearson correlation analysis implemented in MATLAB).

increases the amount of unnatural amino acid–containing protein 1.5- to 2-fold over feeding for 2 d (**Supplementary Fig. 9**).

To direct unnatural amino acid incorporation in a subset of cells within a tissue, we generated flies containing the heat shock–inducible actin-flipout-GAL4 system[25] and Triple-Rep-F. In ovaries dissected from flies fed amino acid **1** and subjected to limited heat shock, we detected full-length GFP-mCherry-HA in a mosaic pattern that matched the pattern of GFP expression. When fly food was not supplemented with amino acid **1**, the amount of anti-HA staining remained equal to background and did not correlate with the GFP levels detected in cells (**Fig. 3b** and **Supplementary Fig. 10**). These data demonstrate unnatural amino acid incorporation in a subset of cells within a tissue in flies.

In conclusion, we have demonstrated that the *Mm*PylRS/$tRNA_{CUA}$ pair is orthogonal to the synthetases and tRNAs found in flies and can be used to direct the incorporation of several unnatural amino acids into proteins in fly cells, fly embryos and adult flies. We have demonstrated the modular combination of the unnatural amino acid incorporation system with several gene expression methods widely used in fly research and the site-specific incorporation of unnatural amino acids into specific proteins expressed at different developmental stages, within specific tissues and in subsets of cells within a tissue. As the *Mm*PylRS/$tRNA_{CUA}$ pair and its synthetically evolved derivatives can be used to incorporate a growing list of useful unnatural amino acids, these experiments provide a foundation for probing and controlling biological processes in flies with molecular precision.

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References

1. Davis, L. & Chin, J.W. *Nat. Rev. Mol. Cell Biol.* **13**, 168–182 (2012).
2. Liu, C.C. & Schultz, P.G. *Annu. Rev. Biochem.* **79**, 413–444 (2010).
3. Gautier, A., Deiters, A. & Chin, J.W. *J. Am. Chem. Soc.* **133**, 2124–2127 (2011).
4. Gautier, A. *et al. J. Am. Chem. Soc.* **132**, 4086–4088 (2010).
5. Lemke, E.A., Summerer, D., Geierstanger, B.H., Brittain, S.M. & Schultz, P.G. *Nat. Chem. Biol.* **3**, 769–772 (2007).
6. Rubin, G.M. & Lewis, E.B. *Science* **287**, 2216–2218 (2000).
7. Adams, M.D. *et al. Science* **287**, 2185–2195 (2000).
8. Brand, A.H. & Perrimon, N. *Development* **118**, 401–415 (1993).
9. Rubin, G.M. & Spradling, A.C. *Science* **218**, 348–353 (1982).
10. Laski, F.A., Ganguly, S. & Sharp, P.A. *Proc. Natl. Acad. Sci. USA* **86**, 6696–6698 (1989).
11. Garza, D., Medhora, M.M. & Hartl, D.L. *Genetics* **126**, 625–637 (1990).
12. Washburn, T. & O'Tousa, J.E. *Genetics* **130**, 585–595 (1992).
13. Lang, K. *et al. Nat. Chem.* **4**, 298–304 (2012).
14. Nguyen, D.P. *et al. J. Am. Chem. Soc.* **131**, 8720–8721 (2009).
15. Nguyen, D.P., Garcia Alai, M.M., Kapadnis, P.B., Neumann, H. & Chin, J.W. *J. Am. Chem. Soc.* **131**, 14194–14195 (2009).
16. Neumann, H., Peak-Chew, S.Y. & Chin, J.W. *Nat. Chem. Biol.* **4**, 232–234 (2008).
17. Nguyen, D.P. *Unnatural Amino Acid Incorporation via the Orthogonal Pyrrolysyl-tRNA Synthetase–tRNACUA Pair*. PhD thesis, Univ. Cambridge (2012).
18. Chen, P.R. *et al. Angew. Chem. Int. Edn. Engl.* **48**, 4052–4055 (2009).
19. Mukai, T. *et al. Biochem. Biophys. Res. Commun.* **371**, 818–822 (2008).
20. Greiss, S. & Chin, J.W. *J. Am. Chem. Soc.* **133**, 14196–14199 (2011).
21. Isogai, Y., Takada, S., Tjian, R. & Keles, S. *EMBO J.* **26**, 79–89 (2007).
22. Mukai, T., Wakiyama, M., Sakamoto, K. & Yokoyama, S. *Protein Sci.* **19**, 440–448 (2010).
23. Bunch, T.A., Grinblat, Y. & Goldstein, L.S. *Nucleic Acids Res.* **16**, 1043–1061 (1988).
24. Van Doren, M., Williamson, A.L. & Lehmann, R. *Curr. Biol.* **8**, 243–246 (1998).
25. Pignoni, F. & Zipursky, S.L. *Development* **124**, 271–278 (1997).

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Author contributions

A.B., F.M.T. and S.G. designed and performed experiments. K.L. provided compounds. A.B. and J.W.C. wrote the paper with input from all authors.

Competing financial interests

The authors declare no competing financial interests.

Additional information

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