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A Genetically Encoded Fluorescent Probe for Detecting Sirtuins in Living Cells

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Abstract

Sirtuins are NAD⁺ dependent protein deacetylases, which are involved in many biological processes. We now report a novel genetically encoded fluorescent probe (EGFP-K85AcK) that responds to sirtuins in living cells. The probe design exploits a lysyl residue in EGFP that is essential for chromophore maturation, and is also an efficient deacetylation substrate for sirtuins. Analysis of activity in *E. coli* cobB revealed that the probe can respond to various human sirtuins, including SIRT1, SIRT2, SIRT3 and SIRT5. We also directly monitored SIRT1 and SIRT2 activity in HEK293T cells with an mCherry fusion of EGFP-K85AcK, and showed that this approach can be extended to other fluorescent proteins. Finally, we demonstrate that this approach can be used to examine the activity of sirtuins toward additional lysyl posttranslational modifications, and show that sirtuins can act as erasers of HibK modified proteins.

Graphical Abstract

The sirtuin protein family, categorized as class III histone deacetylases (HDACs), depends on NAD⁺ for their activity which differs from other Zn²⁺ dependent HDACs. Since the discovery that the deacetylase activity of yeast sirtuin silent information regulator 2 (Sir2) affects life span,^[1] the sirtuin family has received considerable attention.^[2] Recent studies revealed that sirtuin is implicated in a wide range of cellular processes, such as metabolic control, gene transcription, DNA repair, apoptosis, and nutrient sensing. The misregulation of sirtuin activities is associated with a number of diseases, including cancer, obesity,

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Notes

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cardiovascular disease, vessel inflammation and various neurodegenerative diseases.^[3] As a consequence, there has been interest in the development of chemical probes of sirtuin activity. *In vitro* methods including the use of radiolabeled histones^[4] and mass spectrometry,^[5] have been used to measure the deacetylation activity of sirtuins. More recently, fluorescent probes composed of an acetylated peptide and a fluorophore, have been developed to evaluate sirtuin activities in vitro and in living cells.^[6]

The ability to genetically encode noncanonical amino acids (ncAAs) using orthogonal aminoacyl tRNA synthetase (aaRS)/tRNA pairs has enabled the creation of a number of useful cell biological tools.^[7] For example, the ability of some ncAAs to bind or react with small molecules and metal ions has been exploited to design genetically encoded fluorescent probes for Cu²⁺,^[8] Mn³⁺,^[9] H₂O₂,^[10] H₂S^[11] and ONOO⁻.^[12] Here, we take advantage of the ability of an ncAA to modulate fluorescent protein maturation to generate a probe for monitoring sirtuin activities in living cells.

A genetically encoded sirtuin probe was designed based on the assumption that there exists in enhanced green fluorescent protein (EGFP) a lysyl residue that is essential for fluorophore maturation, and which can be efficiently deacetylated by a sirtuin when it is mutated to *N*-ε-acetyl-L-lysine (AcK). In the absence of an active deacetylase, the acetylated EGFP mutant will be nonfluorescent, whereas deacetylation by sirtuin will result in formation of fluorescent EGFP (Fig 1A). In *E. coli*, cobB protein is the only sirtuin, and is the predominate deacetylase (YcgC is a recently discovered lysine deacetylase in *E. coli* that doesn't use NAD⁺ or Zn²⁺).^[13] To identify the requisite lysine in EGFP, we mutated 7 out of the 21 lysyl residues in EGFP (including an Arg that can be replaced by Lys)^[14] to AcK. Most of these residues (K3, K26, K79, K85, K113, K112 and R96) are located at the termini of the β barrel scaffold or in the central α-helix bearing the chromophore (Fig S1). Plasmids containing these EGFP amber nonsense mutants and an orthogonal amber suppressor pyrrolysyl-tRNA synthetase mutant (AcKRS)/tRNA^{Pyl} pair specific for AcK^[15] were co-transformed into *E. coli* MG1655 or *cobB*, and the expression of EGFP mutants was induced by IPTG in the presence or absence of 5 mM AcK. As shown in Fig 1B, one EGFP mutant (K85AcK) showed significantly enhanced fluorescence (~20 fold) in *E. coli* MG1655, whereas no fluorescence enhancement was observed in *E. coli cobB*. This result suggests that the fluorescence of EGFP-K85AcK depends on the activity of cobB.

To verify this notion, a C-terminal His-tagged K85AcK mutant of EGFP was expressed in *E. coli* MG1655 and *cobB*, and analyzed by SDS-PAGE gel (Fig S2) and ESI-QTOF mass spectrometry (Fig 1C). The observed mass (27745.12 Da) of the EGFP-K85AcK mutant expressed in *E. coli* MG1655 was consistent with that of the wild type EGFP. The EGFP-K85AcK mutant expressed in *E. coli cobB* was purified from inclusion bodies under denaturing conditions; the observed mass (27807.25 Da, + 62 Da compared to wt EGFP, Fig S3) matched that of full-length EGFP without deacetylation and without formation of the *p*-hydroxybenzylidene-imidazolidone chromophore. This result confirms that K85 is essential for EGFP fluorescence,^[16] and also suggests that K85 is critical for EGFP chromophore formation. Notably, the soluble EGFP mutant and that isolated from inclusion bodies afforded similar yields (~15 mg/L). The kinetics of fluorophore formation for the EGFP-K85AcK mutant were similar to that of another mutant with AcK at a permissive site

(EGFP-K113AcK); after IPTG induction, fluorescence can be observed in 2 – 3 hours, and reaches a plateau at 8 hours (Fig S4). Taken together, this data shows that EGFP-K85AcK acts as a genetically encoded fluorescent probe of *cobB* activity in *E. coli*.

Next we determined whether EGFP-K85AcK can be used to detect the activity of recombinant mammalian sirtuins expressed in *E. coli* *cobB*. The mammalian sirtuin family comprises seven proteins (SIRT1 – SIRT7); all of them were recombinantly expressed in *E. coli* *cobB* under control of a constitutive *glnS* promoter. Recombinant HDAC3 was also included in this study. SIRT1, SIRT2, SIRT3 and SIRT5 all result in significant fluorescence in the presence of 5 mM AcK, consistent with their known deacetylase activity. All other homologs (SIRT4, SIRT6 and SIRT7) including HDAC3 failed to induce an observable fluorescence enhancement. This is not surprising, as SIRT4 has only ADP-ribosyltransferase activity,^[2e] and SIRT7 is an RNA-activated protein lysine deacylase.^[17] Although SIRT6 has both ADP-ribosyltransferase and deacylase activity, it shows a strong preference for large hydrophobic acyl modifications.^[2b]

We also attempted to directly monitor the sirtuin activities in mammalian cells with EGFP-K85AcK.^[18] To this end, we fused EGFP-K85TAG to the C-terminal of mCherry as an internal standard. Plasmids containing mCherry-EGFP(K85TAG) and AcKRS/tRNA^{Pyl} were co-transfected into human embryonic kidney (HEK) 293T cells in the presence or absence of AcK, and microscopic imaging was carried out after 24 hours. An analysis of the green to red fluorescence ratio is shown in Fig 3B. Strong red fluorescence was observable in all cases, while green fluorescence was AcK dependent, and could be suppressed by sirtuin inhibitors, including NAM, EX-527, and the SIRT2-specific inhibitor SirReal2 (Fig 3). SIRT1 and SIRT2 are present in cytoplasm, and SIRT3 and SIRT5 are localized in mitochondria, so the observed green fluorescence is likely attributed to SIRT1 and SIRT2 activity. Also, the Zn²⁺ dependent HDAC inhibitor (SAHA) did not obviously affect the green fluorescence at 1 μ M concentration (Fig 3A). The IC₅₀ of SIRT1 inhibition by EX-527 was determined to be 6 μ M by microscopic imaging (Fig S5). As a control, the green fluorescence of EGFP fusion with AcK at a permissive site (Y39) was still AcK dependent, but could not be suppressed by sirtuin inhibitors (NAM, EX-527 and SirReal2, Fig S6). The effect of NAM on the fluorescence of mCherry-EGFP-K85AcK and mCherry-EGFP-Y39AcK was further validated by flow cytometry analysis (Fig S7).

Because K85 in EGFP is a conserved residue in other fluorescent proteins derived from either jellyfish *Aequorea victoria* or coral *Discosoma* sp., this AcK replacement strategy may be applicable to other fluorescent proteins with different emission characteristics. To test this notion, an mCherry mutant with the corresponding Lys (K88) mutated to AcK was expressed in *E. coli*. As shown in Fig 4A, the AcK dependent fluorescence enhancement (~25 fold) was inhibited by NAM in *E. coli* MG1655, and no fluorescence enhancement was observed in *E. coli* *cobB*. A C-terminal His-tagged mCherry-K88AcK was then expressed in either MG1655 or *cobB* strains and purified; ESI-QTOF mass spectrometry confirmed the deacetylation of mCherry-K88AcK in *E. coli* MG1655, and the formation of unprocessed inclusion bodies in *E. coli* *cobB* (Fig S8). Sirtuin dependent expression of mCherry-K88AcK in HEK293T cells was also demonstrated by microscopic imaging (Fig S9).

Sirtuins have a broad substrate selectivity,^[2b] and it is likely that the EGFP-K85AcK design concept can be further exploited to investigate sirtuin activity against other Lys posttranslational modifications (PTMs). Lys 2-hydroxyisobutyrylation is a recently discovered histone mark,^[19] and the resulting acylated form of Lys (HibK) has been genetically incorporated into recombinant proteins.^[20] An EGFP mutant with HibK at K85 was expressed in *E. coli* cobB with co-expression of various sirtuin homologs. As shown in Fig 4B, three sirtuin homologs including cobB, SIRT1 and SIRT2 afford fluorescence enhancement in *E. coli* cobB, and this enhancement is inhibited by NAM. Previous work reported that HDAC 1–3 have activity toward HibK modified histones,^[19] and our results indicate that certain sirtuins (especially cobB) can also serve as efficient erasers of HibK modified protein. It's noteworthy that HDAC3 had no effect on EGFP-K85HibK fluorescence in *E. coli* cobB, suggesting a strict context requirement.

In conclusion, we have developed a genetically encoded fluorescent probe for monitoring sirtuin activity in both bacteria and mammalian cells by substituting K85 in EGFP with AcK. K85 is critical for EGFP chromophore maturation, and its acetylated form is also an efficient deacetylation substrate of sirtuin homologs, including cobB, SIRT1, SIRT2, SIRT3 and SIRT5. The probe can be used to assay the activity of recombinant sirtuins in *E. coli* cobB. We also show this design strategy can be extended to other fluorescent proteins with different emission characteristics and is also useful for investigating sirtuin deacylase selectivity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.

A) Design of a genetically encoded EGFP-based sirtuin probe. B) EGFP mutants with genetically encoded AcK at lysine sites were screened by fluorescence in *E. coli* MG1655 and *E. coli* ΔcobB. Fluorescence was measured after 12 hour protein expression with or without 5 mM AcK in LB broth at 37 °C; positive error bars represent standard deviation from the mean (n = 3). The fluorescence was normalized to OD600. C) ESI-QTOF mass spectra of EGFP-K85AcK mutant expressed in *E. coli* MG1655 and *E. coli* ΔcobB.

Fig 2.

EGFP-K85AcK can detect recombinant human sirtuins in *E. coli* cobB. EGFP-K85AcK was expressed in *E. coli* cobB in the presence of constitutively expressed sirtuin homologs and HDAC3. 5 mM AcK was used; the negative control was carried out with an empty pBK2 vector; positive error bars represent standard deviation from the mean ($n = 3$). The fluorescence was normalized to OD600.

Fig 3.

A) Fluorescence imaging of sirtuin activity in HEK293T cells. The cells were co-transfected with two plasmids containing AcKRS/tRNA^{Pyl} and mCherry-EGFP-K85TAG along with addition of different modulators. 4 mM AcK was added 1 hour after transfection. Imaging was carried out after 24 hours. Modulator concentrations: NAM, 10 mM; EX-527, 15 μ M; SirReal2, 15 μ M; SAHA, 1 μ M. Scale bar: 200 μ m. B) The ratiometric value of the corrected total cell fluorescence (CTCF) in the green and red channels. CTCF is defined as the integrated intensity of the whole fluorescence image by ImageJ. Positive error bars represent standard deviation from the mean (n = 3).

A an mCherry-based sirtuin probe

B a platform for sirtuin activity study

Fig 4.

A) mCherry-K88AcK was expressed in *E. coli* MG1655 and *cobB* respectively in the presence or absence of 5 mM AcK. The fluorescence was measured 12 hours after induction with IPTG. 1. MG1655, -AcK; 2. MG1655, +AcK; 3. MG1655, +AcK, 20 mM NAM; 4. *cobB*, -AcK; 5. *cobB*, +AcK. B) EGFP-K85HibK was expressed in *E. coli* *cobB* in the presence of constitutively expressed sirtuin homologs. 2 mM HibK and 20 mM NAM was used in this study. Positive error bars represent standard deviation from the mean ($n = 3$). The fluorescence was normalized to OD600.