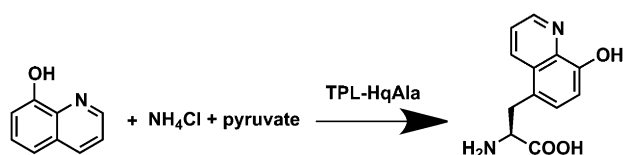


Significant Expansion of the Fluorescent Protein Chromophore through the Genetic Incorporation of a Metal-Chelating Unnatural Amino Acid**

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Genetic incorporation of metal-binding unnatural amino acids^[1] (UAA) is a powerful method for protein sensor design,^[2] metalloenzyme engineering,^[3] and protein NMR spectroscopy.^[4] However, this method is currently underused by chemical biologists, because of the complex synthetic routes to UAAs. Herein, we report a one-step, high-yield enzymatic route for the synthesis of a novel UAA bearing an 8-hydroxyquinoline group (HqAla, Scheme 1), which forms



Scheme 1. Biosynthetic route to HqAla, catalyzed by the tyrosine phenol lyase (TPL) double mutant M288S/F448C.

highly stable complexes with most transition metal ions.^[5] By substituting the Tyr residue of the fluorophore in diverse fluorescent proteins (FPs) with HqAla, we show for the first time that UAA incorporation can result in significantly red-shifted excitation and emission spectra. We solved the crystal structure of superfolder GFP^[6] (sfGFP) with HqAla in its chromophore, revealing the formation of a novel 8-hydroxyquinolin-imidazolinone (HQI) chromophore (Figure 2),

which has a significantly larger conjugated π -system in comparison to the parental 4-(*p*-hydroxybenzylidene)-5-imidazolinone (HBI) chromophore found in *Aequorea victoria* green fluorescent protein (GFP). Our results indicate that HqAla incorporation into the FP fluorophore gives it unique metal-chelating and metal-ion-sensing abilities. Among all biologically relevant metal ions, only Zn^{II} ion binding to HQI causes a significant increase (7.2-fold) in fluorescence. This selective turn-on FP sensor was then applied for Zn^{II} ion sensing in vivo.

We first attempted to transform 8-hydroxyquinoline, a bidentate chelating agent, to 2-amino-3-(8-hydroxyquinolin-5-yl)propanoic acid (HqAla) by using the wild-type *Citrobacter freundii* (ATCC8090) tyrosine phenol lyase (wt TPL), because it has previously been reported that wt TPL has a relatively broad substrate scope.^[3d,7] However, we could not detect any formation of HqAla, using ninhydrin thin-layer chromatography (TLC; Figure 1 B, lane 1). Inspection of the

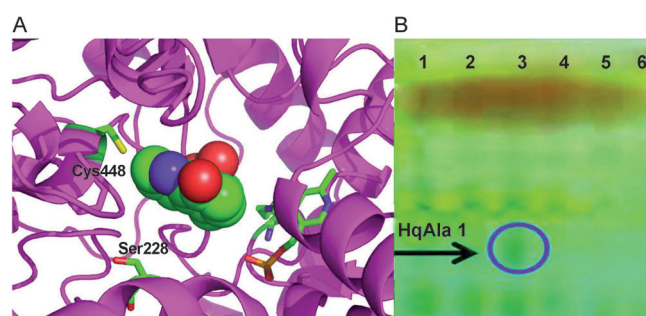


Figure 1. A) Schematic view of the active site model of TPL mutant M288S/F448C (constructed based on wt TPL, PDB code: 2TPL) with HqAla substrate and a cofactor, pyridoxal-5'-phosphate (PLP). Residues Cys448, Ser228, and the PLP cofactor are shown as sticks. The HqAla substrate is shown as spheres. B) Thin-layer chromatography (TLC) assay for TPL-catalyzed HqAla synthesis, stained with ninhydrin. Lane 1: wild-type TPL. Lanes 2-6: TPL mutants with random mutations in residues 36, 288, and 448.

TPL structure showed that residues Phe448, Phe36, and Met288, which together form a hydrophobic pocket and stabilize the tyrosine substrate through van der Waals interactions, can better accommodate the bulky, bicyclic 8-hydroxyquinoline substrate when mutated. To evolve a TPL mutant that can efficiently catalyze the synthesis of HqAla, a TPL library, pEt-TPL2 was constructed. Residues Phe448, Phe36, or Met288 were randomized using an overlapping-

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extension PCR method with synthetic oligonucleotide primers in which the intended randomization sites were encoded by NNT (N = A + T + C + G), affording the library pEt-TPL2 with a size of 4096 members. 1024 clones expressing different TPL mutants were then picked from a petri dish, transferred to liquid minimal medium in microtiter plates, and allowed to grow to saturation. Cells were then lysed with lysozyme, and 8-hydroxyquinoline, NH_4Cl , and pyruvate were added. The plates were then incubated at 37 °C for four hours. Each well of the microtiter plate was then analyzed for amino acid formation by a ninhydrin TLC assay (Figure 1B). We found that one clone (Figure 1B, lane 3) efficiently catalyzed the synthesis of an amino acid. DNA sequencing revealed that this clone had two mutations, M288S and F448C (termed TPL-HqAla). Molecular modeling (Figure 1A) indicated that these mutations, M288S and F448C, result in a significant enlargement of the enzyme pocket to allow for efficient interactions between the enzyme and the substrate, 8-hydroxyquinoline (Figure 1A). Experiments are ongoing to elucidate the structural basis for the efficient transformation of 8-hydroxyquinoline to HqAla by TPL-HqAla. We then scaled up TPL-HqAla expression and performed 8-hydroxyquinoline transformation in gram quantities. The resulting amino acid was separated by HPLC (Supporting Information, Figure S1), and was confirmed to be HqAla by NMR spectroscopy and mass spectrometry (Figure S2). The yield after HPLC purification was 40%. Because all reagents used for the synthesis of HqAla are cheap and environmentally benign, and no organic solvent is used, this new metal binding UAA HqAla can be easily prepared in any laboratory. By contrast, previously reported syntheses of metal-chelating UAAs consisted of more than five steps, required heavy-metal catalysts, carcinogenic solvents, strong acids, and multiple column purification steps, and the final UAA products were in racemic mixtures.^[1b,e]

To selectively incorporate HqAla at defined sites in proteins in *E. coli*, a mutant *Methanococcus jannaschii* tyrosyl amber suppressor tRNA (*MjtRNA*^{Tyr}_{CUA})/tyrosyl-tRNA synthetase (*MjTyrRS*) pair was evolved that uniquely incorporates HqAla in response to the TAG codon, as previously reported.^[3] One *MjTyrRS* clone emerged after three rounds of positive selections and two rounds of negative selections (Table S1). This clone grew at 100 $\mu\text{g mL}^{-1}$ of chloramphenicol in the presence of 1 mM HqAla, but only at 20 $\mu\text{g mL}^{-1}$ chloramphenicol in its absence and was named HqAlaRS. DNA sequencing of this clone revealed the following mutations: Y32H, I63V, L65H, H70G, F108R, Q109V, D158N, L162D, V164G (Table S2). The H70G, I63V, V164G, Q109V mutations create additional space to accommodate the bulky 8-hydroxyquinoline group; Y32, L65, F108, D158, L162 were mutated to hydrophilic amino acids, which creates a larger binding pocket while also

providing additional hydrogen-bonding interactions to stabilize the 8-hydroxyquinoline side chain (Figure S10).

To determine if HqAla can be incorporated into proteins with high efficiency and fidelity, an amber stop codon was substituted for Tyr66 in sfGFP. Protein expression was carried out in *E. coli* in the presence of the selected synthetase (HqAlaRS), *MjtRNA*^{Tyr}_{CUA}, and 1 mM HqAla, or in the absence of HqAla as a negative control. Analysis of the purified protein by SDS-PAGE showed that full-length sfGFP was expressed only in the presence of HqAla (Figure S3), indicating that HqAlaRS was specifically active for HqAla but inactive for the natural amino acids. The yield for the Tyr66→HqAla mutant of sfGFP (termed sfGFP-66-HqAla) was 20 mg L^{-1} . For comparison, the yield of sfGFP was 100 mg L^{-1} . ESI-MS analysis of sfGFP-66-HqAla gave an observed average mass of 27342 Da, in agreement with the calculated mass of sfGFP-66-HqAla (27342 Da), where the chromophore has fully matured after undergoing self-catalyzed dehydration and oxidation reactions (Figure S4). Consistent with this, sfGFP-66-HqAla exhibits fluorescence excitation and emission maxima at 537 nm and 544 nm, respectively (Figure 2), which are red-shifted more than 30 nm compared to those of sfGFP, indicating the formation of an expanded π -system. The excitation and emission spectra of the unnatural amino acid HqAla do not have any

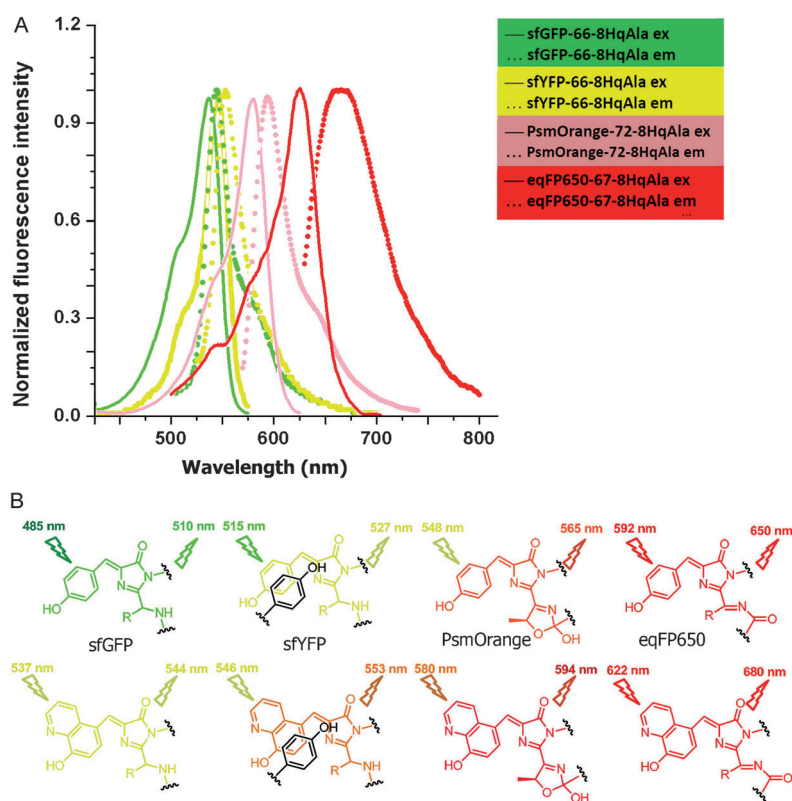


Figure 2. A) Absorption and emission spectra of sfGFP, sfYFP, PsmOrange, and eqFP650 with HqAla in their chromophores. B) Top: chemical formulas of sfGFP, sfYFP, PsmOrange, and eqFP650 chromophores. Bottom: chemical formulas of sfGFP-66-HqAla, sfYFP-66-HqAla, PsmOrange-72-HqAla, and eqFP650-67-HqAla chromophores. Excitation and emission maxima of each chromophore are shown on left and right sides, respectively.

significant overlap with those of sfGFP-66-HqAla (Figure S7), therefore the red-shift seen in the spectra is not due to unincorporated HqAla sidechains.

To elucidate the structural basis for the red-shifted absorption and emission spectra of sfGFP-66-HqAla, we solved its crystal structure at 3.0 Å resolution. Although the substitution of Tyr66 by HqAla causes substantial crowding around the chromophore (Figure 3), the protein backbone of

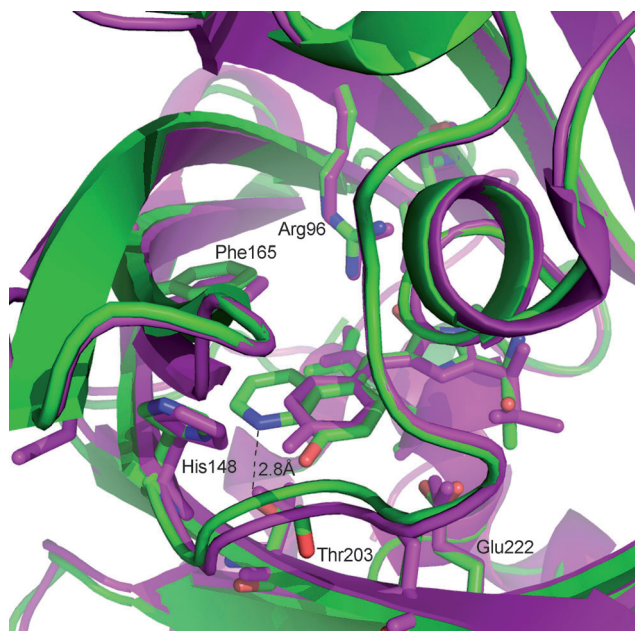


Figure 3. Structural alignment of sfGFP-66-HqAla (green) with sfGFP (magenta, PDB code: 2B3P).

sfGFP-66-HqAla is superimposable with that of sfGFP. Residues His148 and Phe165 are significantly shifted to accommodate the enlarged HQI fluorophore. Phe165 forms π -stacking interactions with HQI, which may reduce the excited-state energy and account in part for the red-shifted absorption and emission spectra. The 8-hydroxyquinoline ring and imidazolinone ring are clearly co-planar, indicating that similar to GFP, nucleophilic attack of the α nitrogen of Gly67 on the carbonyl group of Ser65 has occurred, followed by dehydration and oxidation of the α - β bond of HqAla66 (Figure S4). Residues Arg96 and Glu222, which are essential for the autocatalyzed chromophore maturation in GFP, occupy identical positions in sfGFP-66-HqAla as in sfGFP, indicating that the chromophore-maturation mechanisms of HQI and HBI are highly similar (Figure S4). The FP absorbance and emission spectra are determined by the chemical structure of the chromophore and its surrounding environment. Replacement of the tyrosine residue in the FP chromophore by a tryptophan or other unnatural amino acid without a *para*-hydroxy group, does not result in a significant red-shift of the absorbance/emission spectra.^[2a-c,j] This indicates that the red-shift produced by HqAla is due to the phenol moiety, in addition to having extended π -conjugation.

Since the original discovery of GFP,^[8] significant efforts have been dedicated to the cloning and engineering of GFP-

like proteins with red-shifted excitation/emission spectra.^[2] Such red-shifted GFP variants are extremely useful for tracking protein localization in cells, deep-tissue imaging, and the construction of fluorescence resonance energy transfer (FRET) sensors for a host of signaling molecules and post-translational modifications (PTM).^[2] For example, yellow fluorescent proteins (YFP) which have a Thr203Tyr substitution, form π -stacking interactions between Tyr203 and the HBI chromophore, resulting in a significantly red-shifted spectra^[8] (Figure 2). Moreover, extension of the HBI-conjugated π -system by an imine group (as in mOrange^[9] and PsmOrange^[2f]) or an acylimine group (as in DsRed, eqFP650^[2e]) leads to a more dramatic red-shift of the excitation/emission spectra (Figure 2). FPs that fluoresce in the near-infrared region have lower autofluorescence and lower light scattering; these properties are essential for deep-tissue imaging. To determine if the genetic incorporation of HqAla into various FP chromophores can result in a significant bathochromic shift in general, we substituted Tyr66 of superfolder YFP (sfYFP),^[2i] Tyr72 of PsmOrange, and Tyr67 of eqFP650 (sequences are listed in the Supporting Information) with HqAla. Consistent with the notion that a larger number of conjugated double bonds correlates with longer absorbance/emission wavelengths of FPs, sfYFP-66-HqAla, PsmOrange-72-HqAla, and eqFP650-67-HqAla, each exhibit excitation/emission maxima that are shifted more than 30 nm in comparison with their parental FPs. Remarkably, eqFP650-67-HqAla exhibits excitation and emission maxima at 622 nm and 680 nm, respectively. To our knowledge, eqFP650-67-HqAla has the most far-red excitation peak of any GFP-like fluorescent protein reported to date. In contrast to conventional cloning and directed evolution methods, which can be very time-consuming, our strategy is straightforward for obtaining red-shifted FP variants. While the HqAla FP mutants are relatively dim (Table S3), we are currently optimizing their brightness through random mutagenesis and directed evolution. Further optimization around the chromophore through rational design or high-throughput screening should afford FP variants with further red-shifted excitation/emission spectra. These mutants could be extremely useful for FRET sensor design, and cellular/organismal imaging.

Because HqAla is a metal-chelating UAA, we next examined the metal-ion binding properties of sfGFP-151-HqAla, as we have previously reported for another UAA variant, GFP-151-pyTyr.^[3e] We choose residue 151 because it is not sterically hindered by other residues, and it is relatively close to the GFP chromophore (about 11 Å, Figure S11), therefore Cu^{II} binding to this position might result in significant fluorescence quenching owing to photoinduced electron transfer. We measured the relative fluorescence intensity of 1 μ M sfGFP-151-HqAla in the absence and presence of 1 μ M Cu^{II} ions in 60 mM Tris-HCl, pH 7.0 buffer. Addition of 1 μ M Cu^{II} ions resulted in a 65 % quenching of fluorescence (λ_{ex} = 537 nm; λ_{em} = 544 nm), suggesting that photoinduced electron transfer (PET) from the GFP chromophore to the UAA-bound Cu^{II} ion occurs, resulting in fluorescence quenching. The fluorescence intensity decreased linearly when Cu^{II} ion concentration increased from 0–1 μ M

(Figure S8), but the addition of more than $1\ \mu\text{M}$ Cu^{II} did not result in significantly more quenching. This result indicates that a 1:1 complex was present between sfGFP-151-HqAla and the Cu^{II} ion. We then performed competitive metal-capture analysis by measuring the fluorescence intensity of sfGFP-151-HqAla/ Cu^{II} in the presence of various Cu^{II} chelators with different binding affinity (histidine, bipyridine, *N*-(2-hydroxyethyl)iminodiacetic acid (HIMDA), ethylenediamine-*N,N'*-diacetic acid (EDDA), and ethylenediaminetetraacetic acid (EDTA)). Our results indicate that there is a high-affinity Cu^{II} binding site in sfGFP-151-HqAla, with a K_{D} of 0.1 fM (Figure S5). This result is consistent with previously determined binding affinity between 8-hydroxyquinoline and Cu^{II} .^[5] Notably, the binding affinity of HqAla for the Cu^{II} ion is more than a million-fold higher than that of pyTyr ($K_{\text{D}}=0.9\ \text{nM}$), which we reported earlier.^[3e] These results indicate that HqAla represents a significant advancement over pyTyr as a PET probe for protein electron transfer, because the HqAla/ Cu^{II} complex is much less likely to be affected by adventitious metal-ion binding in proteins or buffers.

We then tested if sfGFP with HqAla incorporated could be used for Zn^{II} ion sensing in vitro and in vivo. We are particularly interested in Zn^{II} ion concentration because it is important in enzyme catalysis, cellular metabolism, gene expression, and neurotransmission, and tremendous effort has been devoted to the development of selective and sensitive Zn^{II} ion sensors that can be excited by visible light.^[10] However, when $100\ \mu\text{M}$ ZnCl_2 was added to sfGFP-66-HqAla ($2\ \mu\text{M}$) in 60 mM Tris-HCl, pH 7.0 buffer, no significant change in fluorescence was detected. We reasoned that the lack of fluorescence response was due to the tight enclosure of the HQI chromophore within the protein cage, rendering it inaccessible to metal ions, as our sfGFP-66-HqAla structure indicates (Figure 4C). To expose the HQI fluorophore for sensing of the external environment, we employed a circular-permutation strategy^[11] to create cpsfGFP-66-HqAla (Figure 4D). The N- and C-termini of sfGFP-66-HqAla were joined with a hexapeptide linker GGTGGS, connecting the original termini. The cpsfGFP-66-HqAla sequence begins from Met145 of sfGFP, and has a C-terminal His-tag to aid purification. When $100\ \mu\text{M}$ of ZnCl_2 was added to cpsfGFP-66-HqAla ($2\ \mu\text{M}$) in 60 mM Tris-HCl, pH 7.0 buffer, a 7.2-fold enhancement in the fluorescence signal was detected ($\lambda_{\text{ex}}=495\ \text{nm}$; $\lambda_{\text{em}}=540\ \text{nm}$). Interestingly, the absorption maximum was blue-shifted by more than 40 nm from the apo (537 nm) to the Zn^{II} -bound form (495 nm), and the emission maximum was also blue-shifted by 10 nm (Figure S6). Blue-shift of the spectrum upon Zn^{II} ion binding is common for quinoline-based Zn^{II} ion sensors.^[10d] We estimated that cpsfGFP-66-HqAla binding to Zn^{II} ions is relatively weak, with a K_{D} around 50–100 μM (Figure S9).^[5] This could be due to steric hindrance of the protein scaffold, because HqAla66 is located deep in the hydrophobic core of the protein. The binding affinity may be enhanced using random mutagenesis and directed evolution. We then tested the effect on the fluorescence signal of cpsfGFP-66-HqAla on other biologically relevant metal ions ($\lambda_{\text{ex}}=495\ \text{nm}$; $\lambda_{\text{em}}=540\ \text{nm}$). As Figure 4A shows, the addi-

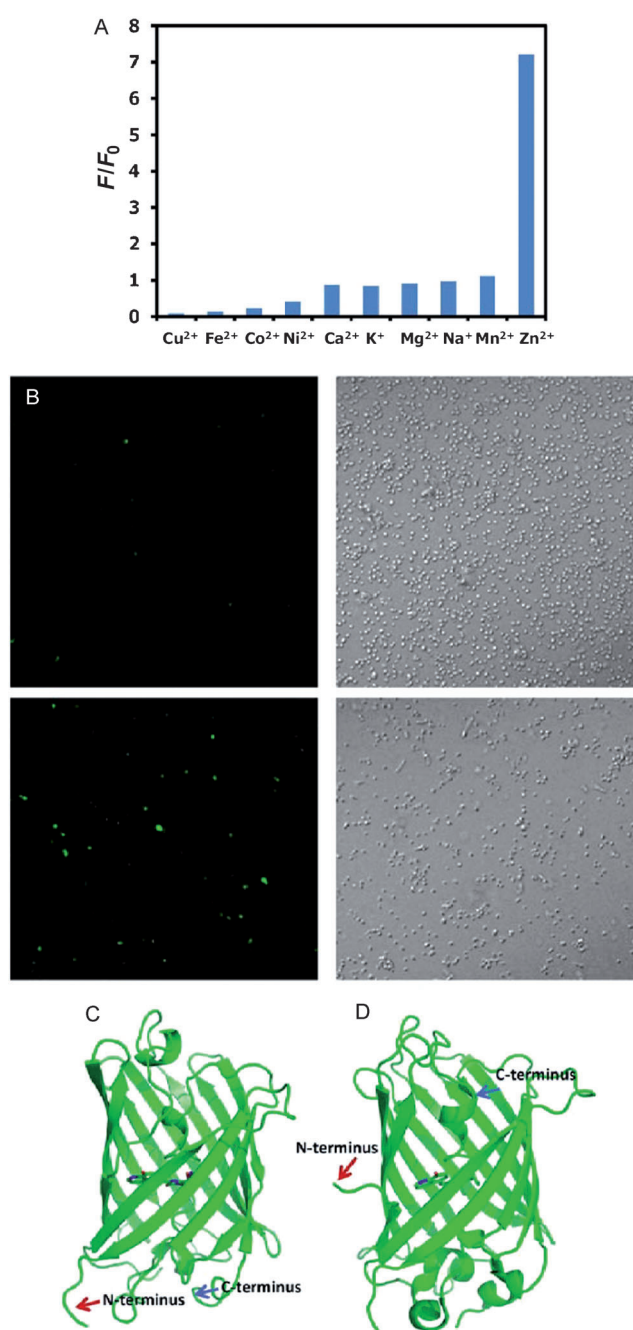


Figure 4. A) Relative fluorescence intensity ($\lambda_{\text{ex}}=495\ \text{nm}$; $\lambda_{\text{em}}=541\ \text{nm}$) of $1\ \mu\text{M}$ cpsfGFP-66-HqAla in the presence of various metal ions ($100\ \mu\text{M}$). B) Confocal fluorescence (left) and bright field (right) images of *E. coli* cells expressing cpsfGFP66-HqAla before (top) and after (bottom) ZnCl_2 ($100\ \mu\text{M}$) treatment. C) Structure of sfGFP-66-HqAla. D) Structural model of cpsfGFP-66-HqAla (built from the crystal structure of cpYFP, PDB code: 3K4).

tion of $100\ \mu\text{M}$ Cu^{II} , Fe^{II} , Co^{II} , or Ni^{II} ions significantly decreased the fluorescence, but other metal ions had little effect. Importantly, only Zn^{II} ions caused a very significant (7.2-fold) increase in fluorescence. We then tested if cpsfGFP-66-HqAla can be used for Zn^{II} ion sensing in vivo. *E. coli* cells overexpressing cpsfGFP-66-HqAla were imaged using a confocal fluorescence microscope, before and after the addition of $100\ \mu\text{M}$ Zn^{II} ions. The cells showed little fluorescence

without addition of Zn^{II} ions and became much more fluorescent after addition of $100\ \mu\text{M}$ Zn^{II} ions (Figure 4B). Our results show for the first time that a genetically encoded metal-chelating UAA can bind Zn^{II} ions in living cells.

In conclusion, we have demonstrated the highly efficient biosynthesis and site-specific incorporation of a new metal-chelating UAA HqAla into proteins. We showed that the genetically encoded metal-chelating UAA HqAla can bind to Cu^{II} ions with sub-femtomolar affinity, and can bind to Zn^{II} ions in living *E. coli* cells. Because the synthetic route of HqAla consists of only one step, with no organic reagents or column purification, HqAla can be easily prepared in any laboratory and mutant proteins bearing HqAla at any site can be easily obtained and purified in milligram quantities by site-directed mutagenesis and recombinant gene expression. Because HqAla can be synthesized through a purely enzymatic route in one step and with high yield, and its 8-hydroxyquinoline motif binds strongly to most transition-metal ions, including lanthanide ions,^[5] we believe that the technological barriers that have limited the application of the genetic incorporation of metal-chelating UAAs have now been overcome. With this new method, the future is now even brighter for GFP engineering, metalloprotein engineering, and protein NMR spectroscopy using paramagnetic metal ions.

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