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Supporting Information

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for

In vivo Imaging of a Bacterial Cell Division Protein Using a Protease-Assisted Small Molecule Labeling Approach

Souvik Chattopadhyaya, Farhana B. Abu Bakar,
Rajavel Srinivasan, and Shao Q. Yao*

1. Mechanism of site-specific protein labeling

Figure S1. Schematic showing the chemoselective reaction between the cysteine residue at the N terminus of the target protein and thioester-containing small molecule probe, leading to the formation of a covalent protein-probe adduct.

2. Probe synthesis

Full experimental details of the design and synthesis of the probes have been previously reported.^[1] Since the outer membrane of Gram negative bacteria restricts the

diffusion of molecules larger than ~ 650 Da, we kept the size of our probes within this molecular weight limit.^[2]

3. In vitro labeling of benzyl-thioester probe with cysteine proteases

In order to determine site-specific nature of the labeling approach (only toward N-terminal cysteine of a protein), control experiments were carried out with pure cysteine proteases (which possess an internal active-site cysteine residue) that were incubated with different concentration of the TAMRA-thioester probe **2** (5, 10, 20 and 25 μ M) for 30 min at 37 °C and analyzed by in-gel fluorescence imaging. Faint nonspecific labeling of TEV was seen at probe concentrations >10 μ M when samples were analyzed after boiling in DTT-containing SDS-sample buffer. However, we did observe labeling of TEV with 5 μ M of the probe when DTT-free SDS sample buffer was used. It is likely that non-specific labeling of TEV can occur under conditions of high concentration of the probe and/or protein (see section 7B for further discussions).

Figure S2. In-gel fluorescence scanning of pure cysteine proteases after labeling with different concentrations of the TAMRA-thioester probe **2**. (L to R) 5, 10, 20 and 25 μ M. The different proteases are 1) Bromelain; 2) Ficin; 3) Papain; 4) TEV. Samples were analyzed on 12% gels after boiling in DTT-free and DTT-containing SDS sample buffer.

4. Cloning, expression and purification of MBP fusion proteins

Standard cloning procedures were followed for the generation of all expression constructs.^[3] All constructs were verified by DNA sequencing.

Mouse dihydrofolate reductase (DHFR) and glutathione-S-transferase (GST) were expressed as fusions to the maltose binding protein (MBP) using the pMal-c2X vector (NEB, USA). The TEV recognition/cleavage sequence ENLYFQ↓C (P₁' residue was changed from glycine/ serine to cysteine) was appended in the linker region between

MBP and its fusion partner such that TEV cleavage would generate the N-terminal cysteine containing protein. The overall design of the MBP-fusion proteins together with primers and RE sites used is shown as follows :

Figure S3. Schematic representation of the MBP-fusions used in this study.

Protein	Linker excluding TEV sequence (bp)	RE sites	Template	Primers
GST	10	BamHI, HindIII	pGEX-4T-1 (GE Healthcare)	<i>Fwd:</i> 5'- GAA TTC <u>GGA TCC</u> GAA AAT CTG TAT TTT CAG TGC ATG TCC CCT ATA CTA GGT TA -3' <i>Rev:</i> 5'- <u>CAA GCT TGC</u> CTG CAG CTC AGC TAA TTA ACG CGG AAC CAG ATC CGA -3'
DHFR	10	BamHI, HindIII	pQE40 (Qiagen)	<i>Fwd:</i> 5- GAA TTC <u>GGA TCC</u> GAA AAT CTG TAT TTT CAG TGC ATG GTT CGA CCA TTG AA-3' <i>Rev:</i> 5'- <u>CAA GCT TGC</u> CTG CAG CTC AGC TAA TTA TTT CTT CTC GTA GAC TTC-3'

TEV recognition and cleavage site is in *italics*; start and stop codons are in **bold**; RE sites are underlined

For expression, the MBP-fusion proteins were transformed into ER2566 strain (NEB) by heat shock method. Overnight cultures were diluted 1:100 in LB media supplemented with 100 µg/mL Ampicillin and grown at 37°C. At OD₆₀₀ ~0.4, expression was induced by addition of 0.4 mM of IPTG (isopropyl-β -D-thiogalactopyranoside) and

cultures were grown for an additional 3-4 h at 37°C. Both the fusion proteins were soluble, expressed in high yields and the expression was verified by immunoblot analysis with anti-MBP antibody (NEB) and Coomassie blue staining.

For protein purification, cells from 200 ml of cultures grown in LB (containing Ampicillin + 0.2% glucose) were harvested by centrifugation (4000 g, 30 min, 4°C) and re-suspended in column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM DTT) containing 1 mg/mL lysozyme. 1 ml of column buffer was added for each 40 mL of culture volume (1:40 dilution). After incubation on ice for 30 min, cells were lysed by sonication (6X pulses of 15 s each at half maximal power was sufficient). Lysates were clarified by centrifugation at 10 000 g, 30 min, 4°C and loaded onto a column containing 100 µL of amylose resin pre-equilibrated with column buffer. Following incubation for 30 min at 4°C, the resin was washed 5 times with column buffer and the fusion was eluted with 10 mM maltose in column buffer. Fractions containing the desired fusion protein were pooled and maltose was removed by buffer exchange using Amicon columns. The typical protein yield was 10-20 mg/L. This protocol was followed for all the fusion constructs with minor modifications in the wash steps as required.

5. Cloning and expression of TEV

For controlled intracellular processing by TEV, both the TEV and the substrate fusion (MBP-fusions) had to be expressed in the same cell (see Figure S11 for example). For this reason we choose the pACYCDuet-1 (Novagen) as it has a compatible replication with pMal-c2X plasmid and a different selection marker (Chloramphenicol, Cam^R). TEV was amplified from pTL-5495 (ATCC) using the primers 5'-CG CG GAT CCG GGA GAA AGC TTG TTT AAG GG-3' (Fwd) and 5'-ATA GTT TAG CGG CCG CTC AGC TAA TTA ATT CAT GAG TTG AGT CG-3'(Rev) and cloned into the BamHI and NotI sites (underlined) within the MCS of pACYCDuet-1. The region of TEV amplified excluded the last six amino acids- ELVYSQ of the catalytic domain. Since the catalytic domain of TEV has a propensity to cleave itself in vitro between residues 218-219 to yield a truncated enzyme with greatly diminished activity,^[4] a site directed mutagenesis was done to alter the serine at the autoproteolytic site to asparagine (S219N).^[5]

For expression, overnight cultures were diluted in fresh LB media containing 34 µg/mL chloramphenicol and grown to OD₆₀₀~0.4 at which point expression was induced by the addition of 0.4 mM IPTG. Cultures were incubated at 37°C with constant shaking. Aliquots of samples at the indicated time points were collected, washed three times with 1 x PBS, lysed by boiling in SDS-sample dye, run on a 12% gel and detected by immunoblot with anti-His antibody (Qiagen) according to the manufacturer's instructions. Briefly, proteins were transferred onto PVDF membrane, blocked at room temperature for 1 h using anti-His HRP Conjugate blocking buffer (containing 0.1% Tween-20) and incubated with anti-His HRP antibody (1:1500 dilution in anti-His HRP Conjugate blocking buffer) for 1 h at room temperature. At the end of this period, blots were washed thrice for 10 min each time in PBST (PBS containing 0.1% Tween-20) and detected by chemiluminescence using the SuperSignal West Pico kit (Pierce). The 6 X His tag is vector derived and present at the N-terminal of TEV.

Figure S4. Immunoblot analysis of time course of TEV expression in ER2566. The blot was probed with anti-His antibody.

6. In vitro cleavage and labeling of MBP-DHFR with 1

For analysis of in vitro cleavage and labeling of MBP-DHFR, 20 µL reaction mixes were set up comprising of 2 µg of the purified fusion protein, 1 µL of TEV (5U, Invitrogen, USA), 1 µM of fluorescein thioester probe 1 and 1 x TEV buffer (50 mM Tris. HCl, 0.5 mM EDTA, 1 mM DTT) and the volume was made up to 20 µL with water. Samples were incubated at 30°C to allow simultaneous cleavage and labeling. At the indicated time points, reaction was stopped by boiling samples with SDS-loading dye and analyzed by in-gel fluorescence scanning and Coomassie blue staining. N-terminal cysteine DHFR was labeled within 15 min with no labeling of the fusion, TEV or MBP protein even after 2 h.

Figure S5. Time course of in vitro cleavage and labeling of N-terminal cysteine-containing DHFR with **1**. A) Fluorescence image and B) Coomassie blue staining of the same gel. We routinely observed that only < 30 % of the fusion is cleaved in vitro.

7. In vivo labeling experiments

A. Time course for in vivo cleavage of N-terminal cysteine-containing GST in bacteria:

For monitoring of TEV-mediated cleavage of MBP-fusions in vivo, we used MBP-GST as the model protein. Bacterial cells transformed with compatible expression plasmids- pMal-C2X-MBP-GST (*Amp^r*) and pACYC-TEV (*Cam^r*), were grown in LB media supplemented with Ampicillin (100 µg/mL) and Chloramphenicol (34 µg/mL). At OD₆₀₀~0.4, expression for both the fusion and TEV were induced simultaneously by the addition of 0.4 mM IPTG. Aliquots of samples were collected at the indicated time point, pelleted, lysed by boiling in SDS-sample dye and analyzed on a 12% gel by both Coomassie blue staining and immunoblot with anti-GST antibody (Amersham). As shown in Figure S6B, TEV was highly efficient in vivo with 90-95% of the fusion being cleaved at any given time.

Figure S6. A) Coomassie blot analysis of time course of cleavage of MBP-GST in ER2566 cells expressing TEV. Aliquots of cells that express TEV only, MBP-GST only or both MBP-GST and TEV were collected at the indicated time-points and analyzed on a 12% SDS-PAGE. B) Immunoblot analysis of in vivo cleavage of MBP-GST. At any given time, in vivo cleavage proceeds to 90-95% completion. The band seen at 0 h could be due to leaky expression.

B. In vivo labeling of control cells expressing TEV only

To confirm that the labeled protein following in vivo cleavage of MBP-fusions was indeed the target N-terminal cysteine protein and not TEV and/or endogenous proteins, we also labeled TEV under similar conditions (25 μ M of TAMRA-thioester probe **2**) for the indicated time points. No labeling of TEV was seen during this time point when samples were analyzed after boiling in DTT-containing SDS sample buffer. However, we did observe TEV being labeled when samples were analyzed following lysis in DTT-free dye. It is likely that TEV gets labeled under conditions of high protein expression. With further optimization of IPTG concentrations, we were able to substantially reduce the labeling of TEV (compare panel A with C).

Figure S7. Fluorescence in-gel scanning ER2566 cells expressing TEV only following induction with different concentrations of IPTG A) 0.05, B) 0.1 and C) 0.4 mM. Labeling was done with 25 μ M of TAMRA-thioester probes for the indicated time points. Bands corresponding to labeled TEV are indicated with *.

C. Labeling of N-terminal cysteine-containing proteins with other probes

Besides DHFR, we also did a time course for in vivo labeling of GST under identical conditions of growth and probe concentrations. A fluorescent band corresponding to N-terminal cysteine GST was seen within 30 min of cleavage and labeling (Figure S8A). Together with DHFR labeling, this result indicates that the method to label N-terminal cysteine protein is general and can be applied to any target protein that has been suitably modified with the TEV recognition and cleavage sequence at the N-terminal.

Apart from TAMRA-thioester probe, we were also able to demonstrate the successful in vivo biotinylation of DHFR using the biotin-thioester probe. However, in immuno-

blot analysis for biotinylated proteins, we observed an additional band, the endogenous *E. coli* biotinylated protein - the biotin carboxyl carrier protein (BCCP) of acetyl-CoA carboxylase (Figure S8B).^[6] Thus our method provides an easy access to a pool of biotinylated target protein(s) that can potentially be captured from the cellular lysate onto avidin slides for generation of protein microarray in which target proteins are immobilized site specifically via the N terminus.^[7]

Figure S8. A) In-gel fluorescence image for the time course of in vivo labeling of N-terminal cysteine-containing GST with TAMRA-thioester probe **2**. B) Time course for labeling of N-terminal cysteine DHFR with biotin-thioester probe. The endogenous *E. coli* biotinylated protein BCCP is indicated with *.

8. Optimization of labeling conditions

To address the issue of TEV labeling (section 7B), we optimized the conditions of IPTG used for in vivo cleavage and labeling. At high concentration of IPTG, besides the N-terminal cysteine containing target protein, TEV was the major labeled protein. Some non-specific labeling was seen (when samples were analyzed with DTT-free SDS sample buffer) but in all likelihood these intermediates are transient in nature and not real macromolecular labeling. Only the reaction between the thioester moiety of the probe and the N-terminal cysteine results in the formation of a stable peptide bond. This is further validated by the fact that such background labeling was not seen when samples were analyzed after boiling in DTT-containing dye.^[8] Furthermore, the non-specific labeling of TEV was almost absent when induction was done using 0.05 mM of IPTG (Figure S9, Panel C). Even under these conditions, the labeling of the N-terminal cysteine containing target protein was seen within 30 min of in vivo cleavage and labeling

Figure S9. In vivo cleavage and labeling using different concentrations of IPTG. ER2566 cells expressing TEV and MBP-GST fusion were induced with the indicated concentrations of IPTG. Samples collected at different time points were lysed by boiling in both DTT-containing (upper panel) and DTT-free (lower panel) dye and analyzed on a 12% gel. At high IPTG concentrations, besides the N-terminal cysteine containing GST, only non-specific labeling of TEV (indicated with *) contributes to the background. The TEV labeling is completely absent when 0.05 mM of IPTG was used for induction. In all the cases, the labeling of the target protein was seen within 30 min of induction.

9. Cloning and expression of TEVseq-FtsZ-GFPmut1 in ER2566

Figure S10. Schematic representation of the FtsZ-GFPmut1 fusions used. The construct used as negative control does not have the additional cysteine and the TEV recognition and cleavage sequence at the N-terminal.

The green fluorescent protein variant cloned at the C terminus of FtsZ is GFPmut1 which has a highly shifted excitation maxima and folds more effectively than *wt* GFP – properties that contribute to an increased fluorescent intensity.^[9] GFPmut1 was cloned into the EcoRI/XbaI (underlined) site of pBAD/Myc-His B vector (Invitrogen) by using the primers; Fwd: 5'-ggg GAA TTC **ATG** AGT AAA GGA GAA GAA CTT TTC-3' and Rev: 5'-ggg TCT AGA CC TTT GTA TAG TTC ATC CAT GC-3'. FtsZ was then cloned into the XhoI/ PstI sites at the N-terminal of GFPmut1 using the primers:5'-ggg CTC GAG **A** GAA AAT CTG TAT TTT CAG TGC **ATG** TTT GAA CCA ATG GAA C-3' and Rev: 5'-ggg CTG CAG **C** AT CAG CTT GCT TAC GCA GGA ATG C-3'. The fusion was in frame with the vector derived C-terminal epitopes- Myc and 6 X His. Addition of the TEV sequence and/or the cysteine residue at the N terminus after TEV cleavage did not interfere with FtsZ localization (compare Figure 3B main text with Figure S12, Panel A).

For expression and simultaneous cleavage of FtsZ-GFPmut1 proteins, ER2566 cells were grown in M9 media containing 0.4% glycerol. At OD₆₀₀~0.4, protein expression was induced by the addition of 0.2% arabinose (for TEVseq-FtsZ-GFPmut1) and 10 µM IPTG (for TEV). Under our experimental conditions, these concentrations of arabinose and IPTG were found to be optimal for protein expression and labeling (see below). M9 media has been extensively used to study FtsZ gene expression.^[10, 11] Growth in M9 minimal media resulted in an increase in doubling time from ~20 min (in LB) media to ~ 80 min. However, this change in growth rate does not alter the levels of FtsZ as indicated in a previous report by Levin and coworkers. In *E. coli*, the formation of the cytokinetic ring is governed by changes in the polymerization kinetics FtsZ during the course of the cell cycle rather than by oscillations in intracellular concentrations.^[11]

Next, we optimized the FtsZ expression levels such that the Z-ring formation by the FtsZ protein can be observed under our labeling conditions. Since overproduction of FtsZ at levels only 2-7 fold higher than wild type from multicopy plasmids have been known to result in minicell formation,^[12] we used a P_{BAD} vector system for FtsZ expression studies. Unlike strong promoters like P_{lac} or P_{TAC}, P_{BAD} allows tight regulation and expression can be modulated to near endogenous levels with varying concentrations of L-arabinose.^[13] In order to ascertain the expression conditions, we used varying concentrations of L-arabinose ranging from 2 X 10⁻⁵ % (wt/ vol) to 2 X 10⁻¹ % (wt/vol). Immunoblot analysis showed no detectable expression of FtsZ-

GFPmut1 at 2×10^{-4} % (w/v). Fluorescence microscopy of cell populations induced with different arabinose concentrations showed that this is indeed true. Hardly any detectable fluorescence was seen at 2×10^{-4} %. For 2×10^{-3} % - 2×10^{-2} % (w/v) range we found a mixed cell population but the FtsZ-GFPmut1 fusion had a diffused cytosolic distribution (Figure S10C). At 2×10^{-2} % (w/v) concentration, the FtsZ formed spiral instead of rings. Upon further increase of arabinose concentration to 2×10^{-1} % (w/v), we observed Z-ring formation. Hence for all further fluorescence microscopy experiments, we used this concentration of arabinose for induction. Note that this concentration was higher than most that were previously reported in the literature. Under our experimental conditions, the requirement for such a high arabinose concentration can be possibly explained by the followings: 1) though the P_{BAD} promoters allow tight regulation, modulation and moderately high expression levels, it nevertheless behaves in an autocatalytic fashion with individual cells are either fully repressed or fully expressing. Increasing arabinose concentration does not produce a gradation of expression in individual cells but simply increases the percentage of induced cells. This happens randomly from cell to cell in a population and is a largely nonreversible process^[14] and 2) the ER2566 strain used in these studies is *ara*⁺ and hence can lower the concentration of the inducer as they grow since these cells can metabolize arabinose and use it as a carbon source.^[15]

Figure S11. A) Immunoblot analysis of dose dependent induction of TEVseq-FtsZ-GFPmut1 expression. Cells were grown in M9 media containing 0.4% glycerol and induced with the indicated arabinose concentrations (L to R) **U**=Un-induced; **1**= 2×10^{-4} ; **2**= 2×10^{-3} ; **3**= 2×10^{-2} ; **4**= $2 \times 10^{-1} \%$ (w/v). No detectable expression was seen with arabinose concentrations $< 2 \times 10^{-3} \%$ (w/v). B) Immunoblot analysis for TEV expression in cells grown in M9 media and induced with different concentrations of IPTG (L to R: Un-induced, 0.01, 0.02, 0.05, 0.1, 0.2, 0.4 mM). Optimal conditions of IPTG and arabinose used are boxed. C) DIC and fluorescence micrographs of TEVseq-FtsZ-GFPmut1 expression in ER 2566 cells grown in M9 media with glycerol as carbon source and induced with the indicated concentrations of arabinose. Z-ring formation was observed only upon induction with 0.2 % (w/v) arabinose. Scale bar = 5 μ m.

10. Bacterial Imaging and FRET measurements

Figure S12. Schematic representation of A) positive cells and B) control cells used for imaging experiments. Both cell types contain plasmid that express TEV and FtsZ-GFPmut1 proteins with the exception that in positive cells the FtsZ-GFPmut1 fusion is preceded by the TEV recognition and cleavage sequence (TEVseq) so that following cleavage, the fusion protein has a cysteine residue at the N terminus.

To validate the site-specific labeling, FRET measurements were done using the acceptor photobleaching method in which the acceptor molecule of the FRET pair is photobleached, resulting in brightening (unquenching) of the donor fluorescence. Mean intensities and their corresponding standard deviation were measured in the region of interest (ROI) for both the post-bleach and pre-bleach images using the MetaMorph software and FRET was tabulated using the following formula.;

$$FRET = 1 - \left(\frac{F^{Donor}}{F_{pb}^{Donor}} \right)$$

$$\text{where, } F^{Donor} = \left(Donor_{gv}^{Pre-bleach} - Background_{gv}^{Pre-bleach} \right)$$

$$F_{pb}^{Donor} = \left(Donor_{gv}^{Post-bleach} - Background_{gv}^{Post-bleach} \right)$$

gv = intensity as gray value

Background= background intensity as gray value

To ascertain that labeling occurs specifically at the N-terminal cysteine of FtsZ, we used control cells that express both FtsZ-GFPmut1 (without the TEV sequence) and TEV and labeled them under identical growth conditions and probe concentrations. Individual cells were examined by fluorescence microscopy (Figure 8A). Some non-specific/background labeling seen was mostly localized to the cell poles. FRET measurement in these regions showed no change in donor intensities following photo-bleaching of the acceptor while for positive controls, we routinely observed 7-10% increment in donor intensities. Cells that express GFPmut1 in the same expression system were always uniformly fluorescent indicating a cytosolic distribution. This further shows that the localization of FtsZ-GFPmut1 fusions is due to targeting by FtsZ to the internucleoid regions. No labeling was seen in cells that express GFPmut1 only or TEV upon addition of 10 μ M of the TAMRA-thioester probe (Figure S12B and C).

Figure S13. Labeling of ER2566 bacterial cells grown in M9 media together with 10 μ M of TAMRA-thioester probe. A) Cells that express both FtsZ-GFPmut1 and TEV; B) TEV only; C) GFPmut1 only. (L to R) DIC mode, GFP, TAMRA channel. GFPmut1 by itself shows a diffused cytosolic distribution. Addition of FtsZ causes it to localize at the sites of cell division (compare panel C with A). Some background labeling can be seen at the cell poles in TAMRA channel for cells expressing both FtsZ-GFPmut1 and TEV (arrowheads in panel A). Scale bar =5 μ m

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