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Hepta-Mutant *Staphylococcus aureus* Sortase A (SrtA_{7m}) as a Tool for *in Vivo* Protein Labeling in *Caenorhabditis elegans*

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Abstract

In vivo protein ligation is of emerging interest as a means of endowing proteins with new properties in a controlled fashion. Tools to site-specifically and covalently modify proteins with small molecules, peptides, or other proteins in living cells are few and far between. Here, we describe the development of a *Staphylococcus aureus* sortase (SrtA)-based protein ligation approach for site-specific conjugation of fluorescent dyes and ubiquitin (Ub) to modify proteins in *Caenorhabditis elegans*. Hepta-mutant SrtA (SrtA_{7m}) expressed in *C. elegans* is functional and supports *in vitro* sortase reactions in a low-Ca²⁺ environment. Feeding SrtA_{7m}-expressing *C. elegans* with small peptide-based probes such as (Gly)₃-biotin or (Gly)₃-fluorophores enables *in vivo* target protein modification. SrtA_{7m} also catalyzes the circularization of suitably modified linear target proteins *in vivo* and allows the installation of F-box domains on targets to induce their degradation in a ubiquitin-dependent manner. This is a noninvasive method to achieve *in vivo* protein labeling, protein circularization, and targeted degradation in *C. elegans*. This technique should improve our ability to monitor and alter the function of intracellular proteins *in vivo*.

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information

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Figures showing immunoblot analysis, SrtA_{7m}-HA expression, protein labeling, small nucleophile reaction substrates, and inducible protein degradation. (PDF)

A table showing *C. elegans* proteins containing an LPXTG motif. (XLSX)

A table showing hypothetical fusion proteins. (XLSX)

The authors declare no competing financial interest.

INTRODUCTION

Sortase A transpeptidases (SrtA) are a class of enzymes that covalently anchor proteins to the cell wall of Gram-positive bacteria.^{1–3} *Staphylococcus aureus* SrtA recognizes proteins that contain an LPXTG motif (where “X” indicates any amino acid) and cleaves the peptide bond between threonine and glycine; the thiol group of the catalytic cysteine serves as the nucleophile.^{4,5} Upon cleavage and concomitant formation of an acyl-enzyme intermediate, the substrate is subsequently linked covalently to an incoming nucleophile; typically, this occurs via the terminal amine of free glycines present in building blocks that participate in the formation of the peptidoglycan layer in Gram⁺ bacteria.^{6,7} Sortase reactions are reversible, as the reaction regenerates a nucleophile byproduct that can engage in ligation reactions that regenerate the initial, unmodified substrate. To drive a sortase reaction to completion, the incoming nucleophile must be present in molar excess over the substrate or the ligation product must be removed from the reaction environment.

Due to its ability to site-specifically link proteins or peptides through peptide bond formation, SrtA is now widely used in protein engineering applications. Recombinant SrtA enables the site-specific modification of peptides, proteins, antibodies, or polymers with a variety of ligation partners, including fluorescent dyes, oligosaccharides, biotin, nucleic acids, glycolipids, or other peptides^{8–13} (Figure 1). The requirements for *in vitro* substrate ligation are the presence of a LPXTG motif in the substrate and an excess of incoming nucleophile in the reaction, typically GGG-(G₃) or GGGGG-(G₅) labeled molecules, e.g., G₃-biotin or G₃-Alexa647. Depending on substrate, nucleophile design, and source of the sortases used, this method allows substrate ligation at the N-terminus, C-terminus, or both.^{9,14–16}

S. aureus SrtA is a Ca²⁺-dependent enzyme that is not functional when expressed in the cytoplasm.^{17,18} However, *Staphylococcus pyogenes* SrtA, a Ca²⁺-independent enzyme that catalyzes the same reaction, can be used to substitute for *S. aureus* SrtA in reaction environments with low Ca²⁺ levels. Indeed, *S. pyogenes* SrtA enabled site-specific cell-surface and intracellular protein labeling in low Ca²⁺ settings, demonstrating its versatility in covalently linking substrates and nucleophiles *in vivo*.^{11,18}

The means to manipulate or visualize signaling networks *in vivo* are critical to increase our understanding of cellular signaling and organismal development. Because the nematode *Caenorhabditis elegans* is transparent, many cellular and organismic processes can be monitored *in vivo* without the need for invasive procedures. For the visualization of proteins

in vivo, worms are injected with cDNA that encodes the target protein genetically fused to either a fluorescent protein, such as GFP, or to a SNAP–CLIP tag that can be specifically labeled with synthetic probes *in vivo*.^{19–22} However, each of these modifications adds approximately 20–25 kDa to the size of the target protein and can perturb structure, stability, or activity of the fusion partner or some combination of all three. Thus, there is a need for new methods that allow site-specific *in vivo* labeling of proteins while minimizing interference with that protein's function.

The ability of SrtA to catalyze intramolecular protein circularization as well as the formation of protein–protein fusions in living cells prompted us to explore *in vivo* applications of sortase to more complex systems, such as intact organisms. We examined the potential of *in vivo* sortagging in *C. elegans*. A Ca²⁺-independent mutant version of *S. aureus* SrtA (SrtA_{7m}) is functional when expressed in *C. elegans*. It allows for sortase-dependent ligation of LPETG-tagged proteins with small molecule nucleophiles or proteins containing N-terminal oligoglycines (G₃ or G₅). Feeding small molecule nucleophiles to *C. elegans* enables sortase-dependent *in vivo* modification of LPETG-tagged proteins. *In vivo* expression of sortase in *C. elegans* can also catalyze the circularization of a suitably modified linear precursor of GFP and enables the rapid degradation of LPETG-tagged proteins through fusion with a G₃-F-box domain. Together, we propose *in vivo* sortagging as a novel strategy by which to site-specifically modify LPETG-tagged proteins in *C. elegans* in an inducible manner.

RESULTS AND DISCUSSION

Lysates of *C. elegans* Expressing SrtA_{7m} Showing Sortase Activity *in Vitro*

Is it possible to use sortase-mediated protein labeling *in vivo* to site-specifically modify proteins in *C. elegans*? By germline injection, we generated a transgenic *C. elegans* strain that contains an extrachromosomal array encoding HA-tagged *S. aureus* hepta-mutant SrtA (SrtA_{7m}) under the control of a heat shock promoter (*phsp-16.2::srtA_{7m}; pmyo-3::mCherry*). This particular mutant version of sortase was engineered to enhance its catalytic activity while simultaneously rendering the enzyme independent of Ca²⁺.^{23–25} To obtain maximal sortase expression upon induction, we heat-shocked approximately 1000 adult animals per sample for 5–180 min at 37 °C and tested intracellular SrtA_{7m} levels 5–1080 min post-induction (Figure S1A,B). We observed robust SrtA_{7m} expression following a short heat pulse of 15 min, and SrtA_{7m} expression levels plateaued 240 min after induction. To visualize sortase expression within the worm, we first heat-shocked SrtA_{7m} as well as N2 animals for 30 min at 37 °C and fixed the animals 30, 90, or 150 min after induction. Microscopic evaluation of these animals confirmed a time-dependent increase in sortase levels and showed that SrtA_{7m} is expressed throughout the animal body except for the germline (Figure S2A). We also observed a strong signal from unhatched eggs, suggesting embryonic expression of sortase upon induction. In contrast, mCherry levels of the *pmyo-3::mCherry* co-injection marker stayed constant (Figure S2B). Based on these results, we set our standard SrtA_{7m} induction condition as 30 min of heat-shock at 37 °C followed by 2 h of recovery at RT. Using these conditions, we first tested if sortase expression alters animal physiology or fitness. We heat-shocked L1, L3, and day 1 adult animals and tested

their lifespan or ability to develop into fertile adults (Figures 2A,B and S2C). Our results showed that sortase expression did not affect lifespan, nor did *srtA*-expressing animals show any changes in body anatomy or experienced delays in development. We also probed if sortase expression would cause acute toxicity that might impair animals for only a few hours prior to recovery. We repeated our tests with day 1 adults and analyzed their motility 4 or 8 h after heat-shock treatment (Figure 2C,D). We did not observe any changes in *C. elegans* motility. SrtA expression in *C. elegans* thus has no obvious negative consequences on animal health. Having established that sortase expression is well-tolerated by *C. elegans*, we evaluated the ability of *C. elegans*-expressed SrtA_{7m} to catalyze a sortase reaction *in vitro* and ascertained the enzymatic activity of *in vivo* expressed SrtA_{7m}. Total worm lysates from SrtA_{7m}-expressing animals were mixed with a purified protein substrate, VHH-LPETG-His₆ and an excess of a triglycine tetramethylrhodamine nucleophile (G₃-TMR) was added. To prevent detergents from interfering with putative SrtA_{7m} activity, worms were lysed using a detergent-free lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl). We analyzed the reaction products by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fluorescent *in situ* gel scanning and detected the formation of the specific conjugate (VHH-TMR) in the reaction mixture, whereas controls from which either precursor substrate proteins or nematode lysate were omitted failed to yield VHH-TMR (Figure S3). Next, we tested whether our labeling method could achieve site-specific biotinylation of a target protein expressed in *C. elegans*. We analyzed *in vitro* reactions combining *C. elegans* (*phsp-16.2::srtA_{7m};peft-3::GFP-LPETG*) lysate with G₃-biotin. This showed successful biotinylation of the GFP-LPETG substrate *in vitro*, confirming that sortase can label substrates coexpressed with the enzyme in *C. elegans* (Figure 3A). We conclude that inducibly expressed SrtA_{7m} in *C. elegans* catalyzes *in vitro* sortase reactions.

SrtA_{7m} Mediation of *in Vivo* Sortase Reactions in *C. elegans*

The successful ligation of small molecule nucleophiles (G₃-TMR or G₃-biotin) to target proteins in complex *C. elegans* lysates prompted us to ask whether sortase could catalyze such reactions also in living nematodes. To exclude the possibility that the nucleophiles intended for use in *in vivo* ligation reactions might intoxicate the nematodes, we first cultured day 1 adult N2 animals for 10 h in liquid nematode growth medium (NGM) supplemented with G₃-biotin (1 mM), G₃-TMR (1 mM), or G₃-Alexa647 (1 mM) and scored their survival after 24 h. Prolonged exposure to high concentrations of nucleophile did not affect survival (Figure S3B). Visual inspection of the animals confirmed that exposed animals were indistinguishable from untreated controls. To then test if sortase could catalyze *in vivo* ligation reactions, we cultured L4 larvae inducibly expressing SrtA_{7m} and GFP-LPETG (*phsp-16.2::srtA_{7m};peft-3::GFP-LPETG*) for 5 h in liquid nematode growth medium (NGM) supplemented with G₃-biotin (1 mM) and analyzed total worm lysates for the presence of the ligation product. GFP-biotin was readily detected in lysates from animals induced for sortase expression, while uninduced controls did not contain the transpeptidation product (Figure 3B). Control samples (mixing control) consisting of a mixture of lysates from strains individually expressing either SrtA_{7m} or GFP-LPETG and fed with G₃-biotin (1 mM) for 5 h in liquid nematode growth medium (NGM). The worm lysis buffer used contained 1% SDS and 2% NP-40 to instantly denature all proteins and prevent ongoing sortase reactions from proceeding. The presence of GFP-biotin was seen only in lysates

from animals that coexpress SrtA_{7m} and GFP-LPETG and that were fed with G₃-biotin (1 mM). We therefore conclude that the biotinylation reaction occurred *in vivo* rather than post-lysis *in vitro*. To test if *in vivo* sortase expression might result in off-target ligation events, we first conducted a bioinformatics analysis to identify all proteins with LPXTG motifs present in the *C. elegans* proteome (Table S1). We identified 92 proteins containing a LPXTG motif; however, none of the motifs were close to either the N- or C-terminus, nor were they embedded within a favorable amino acid stretch that might allow more distant LPXTG motifs to engage in sortase ligations. To address the possibility that endogenous proteins are modified in off-target reactions, we constructed animals that express the substrate protein G₅-GFP-LPETG in a SrtA_{7m}-positive background (*phsp-16.2::srtA_{7m}; pef t-3::G₅-GFP-LPETG*). Using mass spectrometry, we analyzed total lysates from induced animals 4 h after heat-shocking for the presence of unique peptides that would only emerge upon modification of endogenous proteins (Table S2). We did not identify any such peptide, suggesting that sortase expression in *C. elegans* does not result in measurable off-target ligation events. This finding was supported by the absence of any nonspecific bands in our immunoblot-based tests for *in vivo* sortase reactions (Figure S3C).

Next, we tested *in vivo* protein labeling using a different nucleophile, G₃-Alexa647. Successful labeling would allow the detection of sortase reaction products by immunofluorescence microscopy of live animals. A total of 30 adult worms per condition were incubated for 5 h in liquid NGM supplemented with G₃-Alexa647 and then transferred to OP50 plates. After 24 h of incubation, worms were washed off of the plates and analyzed by fluorescence microscopy. In the absence of SrtA_{7m}, G₃-Alexa647 was found to be enriched in the pharynx in 30 out of 30 animals (Figure 3C, panel 2). In contrast, animals that coexpress SrtA_{7m} and GFP-LPETG showed abundant Alexa647 signal in all body parts. The signal partially overlapped with GFP throughout the body (Figure 3C, panel 4) in 28 out of 30 animals, indicating the formation of GFP-Alexa647 conjugates. Biochemical analysis of similarly treated animals confirmed *in vivo* ligation of GFP-LPETG to G₃-Alexa647 (Figure 3D). These results demonstrated the possibility of executing *in vivo* sortase-mediated protein labeling using different probes.

Together, our results represent the first account of successful *in vivo* protein labeling using sortase in *C. elegans*. Feeding worms with synthetically readily accessible small molecules constitutes a simple means of nucleophile administration for sortase reactions *in vivo*.

SrtA_{7m} Enabling of *in Vivo* Protein Circularization in *C. elegans*

Intramolecular circularization confers several advantages to proteins in comparison to the linear form, such as enhanced thermostability and increased resistance to exoproteolytic attack.^{26,27} To evaluate the ability of SrtA_{7m} to promote protein circularization in *C. elegans*, we generated transgenic animals that coexpress SrtA_{7m} and a circularizable GFP variant, G₅-GFP-LPETG (*phsp-16.2::srtA_{7m}; pef t-3::G₅-GFP-LPETG*). Circular GFP species show accelerated mobility on SDS-polyacrylamide gels, while their fluorescence properties are not affected.²⁶ The N-terminal pentaglycine motif of G₅-GFP-LPETG can function as a nucleophile to resolve acyl-enzyme intermediates (G₅-GFP-LPET-SrtA_{7m}), thus enabling sortase-mediated GFP circularization.²⁶ We analyzed total worm lysates of induced and

uninduced *phsp-16.2::srtA_{7m};**pef t-3::G₅-GFP-LPETG* animals by immunoblotting and observed accumulation of faster migrating (circular) GFP species as well as GFP oligomers upon induction of sortase expression (Figure 4A, lanes 1 and 2). Because protein circularization regenerates a LPETG motif at the site of covalent closure, SrtA_{7m} should be able to cleave the circularized species, too. We supplemented *C. elegans* lysates from induced *phsp-16.2::srtA_{7m};**pef t-3::G₅-GFP-LPETG* animals with a large molar excess of purified SrtA_{7m} and G₃-Biotin and observed the conversion of low-molecular-weight cyclic GFP as well as GFP oligomers to linear monomeric GFP species in the presence of both exogenous enzyme and nucleophile (Figure 4A, lanes 3, 4). Quantification of the different reaction products in Figure 4A showed that the sortase reaction products accounted for approximately 30% (29% ± 3%) of the total GFP pool, highlighting the reversibility of the reaction. This confirms that the products sortase-mediated protein circularization can be resolved by sortase in the presence of excess nucleophile. We conclude that SrtA_{7m} can mediate reversible protein cyclization in *C. elegans*.

SrtA_{7m} Allowance of Targeted Degradation of Suitably Modified Substrates in *C. elegans*

To expand the range of applications for *in vivo* sortagging in *C. elegans*, we engineered strains that would allow specific engagement of the Ub-proteasome pathway. We hypothesized that sortase-dependent ligation of an F-box domain onto a target protein of interest should recruit the target to SKP1-CUL1-F-box complexes and to E2 ubiquitin ligases and thus tag the target protein for degradation (Figure 5B). We constructed transgenic strains that either expressed Ced-10-LPETG-MYC driven by an *eft-3* promoter or encoded a nucleophile G₃-F-box-FLAG construct under the control of a heat-inducible promoter. Expression of both constructs was verified by immunoblotting (Figure 5A). Through crossing, we obtained strains that encoded all three components required for F-box installation onto the target protein (*srtA_{7m}*, *ced-10-LPETG-MYC*, G₃-F-box-flag). We observed robust expression of the Ced-10-LPETG-MYC substrate at 20 °C. However, Ced-10-LPETG-MYC levels markedly decreased when these transgenic nematodes were heat-shocked to induce coexpression of the nucleophile G₃-F-box-FLAG and sortase, together with Ced-10-LPETG-MYC (Figure 5C). We conclude that Ced-10-LPETG-MYC is proteolytically degraded upon sortase-dependent F-box attachment. Control samples (*Ced10::SrtA_{7m}* or *Ced10:Fbox*) did not show a significant reduction in total Ced-10-LPETG-MYC levels (Figure 5A,D). We also tested the kinetics of SrtA_{7m}-mediated Ced-10-LPETG-MYC degradation and observed the initiation of degradation as soon as 30 min after heat shock. At 5 h post-induction, the Ced-10-LPETG-MYC protein signal was almost undetectable, indicating near-complete degradation of the substrate (Figure 5E,F). The addition of the proteasome inhibitor (MG-132) to the plates 30 min after heat-shock onset largely inhibited degradation of Ced-10-LPETG-MYC (Figure S4A). The slight decrease in total Ced-10-LPETG-MYC levels can be attributed to early proteolytic events that occurred before intracellular MG-132 levels reached inhibitory concentrations. SrtA_{7m} can therefore catalyze the *in vivo* ligation of a G₃-F-box domain to its designated, LPETG-tagged target in less than 30 min, mediating substrate depletion within 5 h through engagement of the Ub proteasome pathway.

Conclusions

The ability to introduce site-specific covalent protein modifications in a living organism, and to do so at will, poses an experimental challenge. Here, we show that *in vivo* expression of SrtA_{7m} in *C. elegans* enables reliable site-specific protein modification. We demonstrate the versatility of this approach through the labeling of protein targets with small molecules, by showing intramolecular protein circularization as well as protein–protein ligation. We apply this method to attenuate intracellular protein levels in a sortase-dependent manner through engagement of the proteasome-degradation pathway.

Wild-type *S. aureus* SrtA is a Ca²⁺-dependent enzyme that does not support sortase reactions in the low intracellular Ca²⁺ milieu of metazoan cells. Although previous efforts used *S. pyrogenes* SrtA, a Ca²⁺-independent transpeptidase, to substitute for *S. aureus* SrtA in *in vivo* applications, we now expressed a hepta-mutant *S. aureus*, SrtA_{7m}, in *C. elegans*. A pair of mutations (E105 K/E108A) confer Ca²⁺ independence, and the remaining five mutations (P94R/D160N/D165A/K190E/K196T) improve the catalytic properties of the enzyme.^{23–25} We demonstrate the *in vivo* activity of SrtA_{7m}, not only to site-specifically label LPETG-tagged target proteins with distinct G₃-containing nucleophiles but also to promote intramolecular circularization as a second example.²⁸ Feeding sortase-expressing *C. elegans* with G₃-labeled fluorescent dyes, such as G₃-TMR or G₃-Alexa647, resulted in modification of the intended target proteins. Our results indicate that after 5 h of incubation with G₃-Alexa647, the dye is distributed throughout the animal. Although tissue penetration of the G₃ nucleophiles presumably depends largely on active and passive diffusion, the expression of sortase using tissue-specific promoters may create new possibilities for localizing and tracking proteins of interest in different tissues or organelles. Covalent ligation of a biotin handle to LPETG-tagged proteins may offer an alternative to the use of BirA biotin ligase to enrich for targets in immunoprecipitation assays.

As expected, *in vivo* protein labeling reactions using our sortase-based ligation setup in *C. elegans* did not run to completion with the exception of the F-box-target fusions. Sortase reactions using a G₃- or G₅-nucleophile are reversible because upon completion of the reaction, the LPXTG motif is restored. Only the immediate removal of the ligation product from the reaction environment, such as the case for the FBox-target fusion that was readily degraded by the ubiquitin–proteasome pathway,^{29,30} allows the reaction to reach completion. Alternatively, the presence of a large molar excess of the intact nucleophile can drive the equilibrium in favor of the desired reaction product. *In vivo* reactions with both substrate and nucleophile at approximately equimolar concentrations are expected to yield significantly less than 50% ligation product, even after prolonged incubation. Dealing with the reaction's reversibility, for example by using depsipeptides, β -hairpin linkers or a nickel-peptide complex may prove useful to enhance the efficiency of *in vivo* sortase reactions and shift the equilibrium in favor of the intended ligation product.^{13,31–33}

We show that we can specifically deplete a target protein *in vivo* in a sortase-dependent, inducible manner. The proteolytic removal of F-box-target fusions, a reaction product required for reversing the sortase reaction, is expected to drive the reaction to complete substrate turnover, even at low local nucleophile concentrations.

Methods to target a tagged protein for degradation and to do so inducibly in intact cells or organisms are scarce. Armenti and co-workers showed that expression of the E3 ubiquitin ligase substrate recognition unit Zif-1 is sufficient to enable the rapid degradation of ZF-1-tagged proteins in *C. elegans*,³⁴ and Zhang et al. introduced the plant auxin-inducible degradation system (AID) into *C. elegans* and demonstrated reliably depletion of degron-tagged proteins within 30 min in an auxin-dependent manner.³⁵ Compared to these two very efficient proteolytic systems, sortase-dependent target depletion exhibits slower overall reaction kinetics. Considering that it takes approximately 2 h for SrtA to be expressed at maximal levels post induction (Figure S1A), however, the actual ligation-based depletion reaction is only slightly slower than the ZF-1 or auxin-dependent setups. Importantly, the implementation of our sortase-based target depletion system requires the installment of only a five amino acid LPXTG tag on the target protein's N- or C-terminus, while the ZF-1 tag (44 amino acids) and the degron tag (36 amino acids) are fairly large. The relatively small size of the LPXTG tag minimizes the chance for tag-related off-target effects, translational limitations, or changes in overall target protein structure. Unlike the ZF-1 and auxin systems that are specifically designed to decrease target protein levels, sortase mediated protein depletion is only one of numerous applications for sortase-dependent *in vivo* protein ligation, rendering this new method more versatile than other approaches.

In conclusion, we provide a proof of concept for a SrtA_{7m}-based protein modification strategy in living *C. elegans*. The method is specific, robust, and complements existing protein modification and tracking approaches to study protein function in *C. elegans*.

MATERIALS AND METHODS

C. elegans Strains and Growth Conditions

Wild-type N2 Bristol was maintained at 20 °C on nematode growth medium (NGM) plates containing *Escherichia coli* OP50 bacteria as a food source.³⁶ Synchronized worm populations were obtained by bleaching gravid adults washed off NGM plates and culturing the recovered eggs on fresh NGM plates. All strains containing phsp16.2-based arrays were maintained at 15 °C to suppress basal activation of the hsp-16.2 promoter.

C. elegans Strains Used in This Study

N2, MT23063 (nEx2249[P*myo-3*::*mCherry*, P*hsp16.2*::*G5-GFP-LPETG*]), MT23311 (nIs699[P*myo-3*::*mCherry*, P*hsp16.2*::*srtA_{7m}*]), MT23699 (nIs699; nEx2431) MT23698 (nEx2431[P*peft-3*::*GFP-LPETG*]), MT23700 (nIs699; nEx2432[P*peft-3*::Ced-10-LPETG-MYC, P*hsp16.2*::G3-SCFbox-FLAG, pRF4[rol-6(*su1006dm*)]]), MT23701 (nIs699, nEx2433[P*peft-3*::Ced-10-LPETG-MYC, pRF4]), and MT23702 (nEx2432) were used.

Plasmids

To construct heat-inducible plasmids, we used a pPD49.78 plasmid backbone containing a hsp-16.2 promoter linked to a multiple cloning site. The Fire Lab *C. elegans* Vector Kit was a gift from Andrew Fire. Target genes were cloned into *NheI*-*SacI* digested plasmid backbones using the Invitrogen Gibson Assembly kit following the manufacturer's instructions. ORFs encoding for SrtA_{7m}, GFP-LPETG, GGG-GFP-LPETG, Ced-10-

LPETG-MYC, and GGG-F-Box-FLAG were designed and, if required, the codons optimized *in silico*. To optimize gene expression, 51 base pairs (bps) long synthetic introns (gtaagttaaacaatgattttactaactaactaatctgatttaaatttcag) were introduced in one or two copies into all open reading frames (ORFs). ORFs were synthesized (idtDNA) and amplified by PCR to introduce the required flanking restriction sites followed by 20 bps homology overlap using the primers listed in Table 1. Purified PCR products were ligated into NheI–SacI-digested pPD49.78 using Gibson assembly. For constitutive expression, we exchanged *phsp-16.2* in pPD49.78 with a synthetic DNA fragment that encodes *pef t-3*. Table 1 lists plasmids and primers used in this study.

Generation of Transgenic *C. elegans* Strains

Transgenic animals were obtained by germline injection of N2 worms. Plasmids *pmyo-3::mCherry* as well as PRF4 (rol-6(*su1006dm*)) were used as co-injection markers. Array-positive animals were selected, cultured as individual strains, and evaluated. For array integration, 30 L4 animals were exposed to γ irradiation and afterward grown for 3 weeks at 20 °C to allow Dauer larvae to emerge. A total of 50 array-positive Dauer animals were singled out, and resultant strains were tested for genomic array integration and homozygosity by microscopy and Western blotting post heat-shocking.

Heat-Shocking Protocol

To ensure reproducibility, we performed all experiments that required heat shock using a standardized protocol: animals on NGM plates were placed on a preheated metal tray (37 °C) and immediately transferred into a 37 °C incubator. Following 30 min of incubation, plates were removed and immediately spread out on a lab bench in a 20 °C room without stacking. After 3 h of initial recovery, plates were stacked and stored at 20 °C for the indicated times.

Protein Expression in *C. elegans*

For inducible protein expression, synchronized L1 populations were distributed on fresh NGM plates and grown for 24–80 h at 20 °C prior to heat shocking. Worms containing p49.78-based arrays were heat-shocked at indicated larval or adult stages at 37 °C for the indicated times and transferred to room temperature to allow for protein expression. The number of animals per biochemical experiment was set such that the resulting worm pellet available for analysis would have a volume of ~50 μ L, which corresponds to ~1000 adult animals.

Generation of *C. elegans* Lysate

Synchronized *C. elegans* populations were washed off of NGM plates, resuspended in appropriate buffer (lysis buffer: 50 mM Tris, 100 mM NaCl, pH 7.5, 1% SDS, 2% NP40; sortagging buffer: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl) and sonicated for 30 s (intervals of 3 s on and 3 s off at 10% duty cycle control). The lysate was then centrifuged for 10 min at 15000g at 4 °C. Protein concentration was measured by Nanodrop.

In Vitro Sortase Reaction

Synchronized *phsp-16.2::srtA_{7m}; pmyo-3::mCherry* worms were collected, resuspended in approximately 100 μ L of sortagging buffer, and lysed as described above. Total protein concentration in worm lysates was determined by Nanodrop. Transpeptidation reactions were performed by combining worm lysates normalized for protein concentration in the presence or absence of G₃-TMR nucleophile or G₃-biotin (1 mM) followed by incubation at 4 °C overnight. Reactions were terminated by the addition of SDS-PAGE loading buffer, boiled for 5 min, and analyzed by SDS-PAGE.

Immunoblotting

Samples were supplemented with an equal volume of 2 $\times$ SDS-PAGE sample buffer and boiled for 5 min at 95 °C. A total of 20 μ L of each sample was loaded on 15% SDS-PAGE gels (Mini-Gels, Bio-Rad). Gels were run at 100 mV for 20 min and then 150 mV for 40 min. Proteins were transferred to PVDF membranes using a semidry transfer system. Membranes were blocked overnight in PBS–0.1% TritonX–5% milk and then probed with the appropriate antibody (Table 2). Membranes were developed with ECL reagent (PerkinELmer Life Science), imaged on XAR-5 film (Kodak) and processed.

Nucleophile Synthesis and Feeding

G₃-Biotin, G₃-TMR, or G₃-Alexa647 were synthesized, as described in refs 14, 37, and 38. For feeding, nucleophiles in water were diluted to a final concentration of 1 mM in NGM containing living OP50 *E. coli* bacteria as food-source L4 stage larvae or young adult nematodes were added, and animals were incubated for 5 h at RT with gentle shaking. Control worms were grown in plain NGM. Worms were then transferred to the fresh OP50 plates and incubated for at least 12 h before the preparation of lysates.

Microscopy

For fluorescence microscopy, worms at the L3–L4 stage were paralyzed with levamisole on an agar pad. All images were collected on a PerkinElmer Ultraview multispectral spinning disk confocal microscope equipped with a Zeiss 1.4 numerical aperture oil immersion $\times$ 63 objective lens and a Prior piezoelectric objective focusing device. Images were acquired with a Hamamatsu ORCA ER cooled CCD camera controlled with Volocity software. Post-acquisition image manipulations as well as Pearson correlation coefficient calculations were performed using Fiji software.³⁹ Images shown in Figure 3C represent projections of acquired z-stacks.

Typhoon Scanning

To analyze reaction products of *in vitro* sortagging reactions using G₃-TMR or G₃-Alexa647 as nucleophiles, samples were separated by 10% SDS-PAGE, and gels were scanned using a Typhoon scanner (GE healthcare). Excitation and emission filters were set to 555 and 580 nm for TMR, respectively, and 633 and 670 nm for Alexa647, respectively. Gels were stained afterward with InstantBlue to visualize total sample content.

Longevity, Survival, and Motility Assays

For longevity assays, animals were grown on NGM plates and transferred to fresh NGM plates every 2 days for the first 10 days. At least 100 animals per condition were scored. For survival assays, animals exposed to indicated nucleophiles were transferred to NGM plates and incubated for 24 h at 20 °C before assessing. Animals were considered dead if they did not react to 10 pinches with a platinum loop. Motility assays were performed as described in ref 40. In brief, animals were transferred to a 15 μ L drop of M9 and rested for 3 min. Afterward, body bending was scored for 30 s.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Mazmanian SK, Liu G, Ton-That H, Schneewind O. Staphylococcus aureus sortase, an enzyme that anchors surface proteins to the cell wall. *Science*. 1999; 285:760–763. [PubMed: 10427003]
2. Ton-That H, Mazmanian SK, Faull KF, Schneewind O. Anchoring of surface proteins to the cell wall of Staphylococcus aureus. Sortase catalyzed in vitro transpeptidation reaction using LPXTG peptide and NH(2)-Gly(3) substrates. *J. Biol. Chem.* 2000; 275:9876–9881. [PubMed: 10734144]
3. Mazmanian SK, Ton-That H, Schneewind O. Sortase-catalysed anchoring of surface proteins to the cell wall of Staphylococcus aureus. *Mol. Microbiol.* 2001; 40:1049–1057. [PubMed: 11401711]
4. Ilangovan U, Ton-That H, Iwahara J, Schneewind O, Clubb RT. Structure of sortase, the transpeptidase that anchors proteins to the cell wall of Staphylococcus aureus. *Proc. Natl. Acad. Sci. U. S. A.* 2001; 98:6056–6061. [PubMed: 11371637]
5. Kruger RG, Otvos B, Frankel BA, Bentley M, Dostal P, McCafferty DG. Analysis of the substrate specificity of the Staphylococcus aureus sortase transpeptidase SrtA. *Biochemistry*. 2004; 43:1541–1551. [PubMed: 14769030]
6. Popp MW, Ploegh HL. Making and breaking peptide bonds: protein engineering using sortase. *Angew. Chem., Int. Ed.* 2011; 50:5024–5032.
7. Clancy KW, Melvin JA, McCafferty DG. Sortase transpeptidases: insights into mechanism, substrate specificity, and inhibition. *Biopolymers*. 2010; 94:385–396. [PubMed: 20593474]
8. Ritzefeld M. Sortagging: a robust and efficient chemoenzymatic ligation strategy. *Chem. - Eur. J.* 2014; 20:8516–8529. [PubMed: 24954404]
9. Schmohl L, Schwarzer D. Sortase-mediated ligations for the site-specific modification of proteins. *Curr. Opin. Chem. Biol.* 2014; 22:122–128. [PubMed: 25299574]
10. Proft T. Sortase-mediated protein ligation: an emerging biotechnology tool for protein modification and immobilisation. *Biotechnol. Lett.* 2010; 32:1–10. [PubMed: 19728105]
11. Sweet LK, Guimaraes CP, Sehrawat S, Spooner E, Barrasa MI, Ploegh HL. Sortase-mediated modification of alphaDEC205 affords optimization of antigen presentation and immunization against a set of viral epitopes. *Proc. Natl. Acad. Sci. U. S. A.* 2013; 110:1428–1433. [PubMed: 23297227]
12. Popp MW, Antos JM, Grotenbreg GM, Spooner E, Ploegh HL. Sortagging: a versatile method for protein labeling. *Nat. Chem. Biol.* 2007; 3:707–708. [PubMed: 17891153]

13. Antos JM, Truttmann MC, Ploegh HL. Recent advances in sortase-catalyzed ligation methodology. *Curr. Opin. Struct. Biol.* 2016; 38:111–118. [PubMed: 27318815]
14. Theile CS, Witte MD, Blom AE, Kundrat L, Ploegh HL, Guimaraes CP. Site-specific N-terminal labeling of proteins using sortase-mediated reactions. *Nat. Protoc.* 2013; 8:1800–1807. [PubMed: 23989674]
15. Antos JM, Chew GL, Guimaraes CP, Yoder NC, Grotenbreg GM, Popp MW, Ploegh HL. Site-specific N- and C-terminal labeling of a single polypeptide using sortases of different specificity. *J. Am. Chem. Soc.* 2009; 131:10800–10801. [PubMed: 19610623]
16. Dorr BM, Ham HO, An C, Chaikof EL, Liu DR. Reprogramming the specificity of sortase enzymes. *Proc. Natl. Acad. Sci. U. S. A.* 2014; 111:13343–13348. [PubMed: 25187567]
17. Race PR, Bentley ML, Melvin JA, Crow A, Hughes RK, Smith WD, Sessions RB, Kehoe MA, McCafferty DG, Banfield MJ. Crystal structure of *Streptococcus pyogenes* sortase A: implications for sortase mechanism. *J. Biol. Chem.* 2009; 284:6924–6933. [PubMed: 19129180]
18. Strijbis K, Spooner E, Ploegh HL. Protein ligation in living cells using sortase. *Traffic.* 2012; 13:780–789. [PubMed: 22348280]
19. Gautier A, Juillerat A, Heinis C, Correa IR Jr, Kindermann M, Beauflis F, Johnsson K. An engineered protein tag for multiprotein labeling in living cells. *Chem. Biol.* 2008; 15:128–136. [PubMed: 18291317]
20. Gautier A, Nakata E, Lukinavicius G, Tan KT, Johnsson K. Selective cross-linking of interacting proteins using self-labeling tags. *J. Am. Chem. Soc.* 2009; 131:17954–17962. [PubMed: 19916541]
21. Ullrich M, Liang V, Chew YL, Banister S, Song X, Zaw T, Lam H, Berber S, Kassiou M, Nicholas HR, Gotz J. Bio-orthogonal labeling as a tool to visualize and identify newly synthesized proteins in *Caenorhabditis elegans*. *Nat. Protoc.* 2014; 9:2237–2255. [PubMed: 25167056]
22. Machleidt T, Robers M, Hanson GT. Protein labeling with FIAH and ReAsH. *Methods in molecular biology.* 2007; 356:209–220. [PubMed: 16988405]
23. Hirakawa H, Ishikawa S, Nagamune T. Design of Ca²⁺-independent *Staphylococcus aureus* sortase A mutants. *Biotechnol. Bioeng.* 2012; 109:2955–2961. [PubMed: 22729808]
24. Swee LK, Lourido S, Bell GW, Ingram JR, Ploegh HL. One-step enzymatic modification of the cell surface redirects cellular cytotoxicity and parasite tropism. *ACS Chem. Biol.* 2015; 10:460–465. [PubMed: 25360987]
25. Hirakawa H, Ishikawa S, Nagamune T. Ca²⁺-independent sortase-A exhibits high selective protein ligation activity in the cytoplasm of *Escherichia coli*. *Biotechnol. J.* 2015; 10:1487–1492. [PubMed: 25864513]
26. Antos JM, Popp MW, Ernst R, Chew GL, Spooner E, Ploegh HL. A straight path to circular proteins. *J. Biol. Chem.* 2009; 284:16028–16036. [PubMed: 19359246]
27. Popp MW, Dougan SK, Chuang TY, Spooner E, Ploegh HL. Sortase-catalyzed transformations that improve the properties of cytokines. *Proc. Natl. Acad. Sci. U. S. A.* 2011; 108:3169–3174. [PubMed: 21297034]
28. Hirakawa H, Ishikawa S, Nagamune T. Ca²⁺-independent sortase-A exhibits high selective protein ligation activity in the cytoplasm of *Escherichia coli*. *Biotechnol. J.* 2015; 10:1487–1492. [PubMed: 25864513]
29. Caussinus E, Kanca O, Affolter M. Fluorescent fusion protein knockout mediated by anti-GFP nanobody. *Nat. Struct. Mol. Biol.* 2012; 19:117–121.
30. Shin YJ, Park SK, Jung YJ, Kim YN, Kim KS, Park OK, Kwon SH, Jeon SH, Trinh le A, Fraser SE, Kee Y, Hwang BJ. Nanobody-targeted E3-ubiquitin ligase complex degrades nuclear proteins. *Sci. Rep.* 2015; 5:14269. [PubMed: 26373678]
31. Yamamura Y, Hirakawa H, Yamaguchi S, Nagamune T. Enhancement of sortase A-mediated protein ligation by inducing a beta-hairpin structure around the ligation site. *Chem. Commun.* 2011; 47:4742–4744.
32. Yazawa K, Numata K. Recent advances in chemoenzymatic peptide syntheses. *Molecules.* 2014; 19:13755–13774. [PubMed: 25191871]

33. David Row R, Roark TJ, Philip MC, Perkins LL, Antos JM. Enhancing the efficiency of sortase-mediated ligations through nickel-peptide complex formation. *Chem. Commun.* 2015; 51:12548–12551.
34. Armenti ST, Lohmer LL, Sherwood DR, Nance J. Repurposing an endogenous degradation system for rapid and targeted depletion of *C. elegans* proteins. *Development.* 2014; 141:4640–4647. [PubMed: 25377555]
35. Zhang L, Ward JD, Cheng Z, Dernburg AF. The auxin-inducible degradation (AID) system enables versatile conditional protein depletion in *C. elegans*. *Development.* 2015; 142:4374–4384. [PubMed: 26552885]
36. Brenner S. The genetics of *Caenorhabditis elegans*. *Genetics.* 1974; 77:71–94. [PubMed: 4366476]
37. Li Z, Theile CS, Chen GY, Bilate AM, Duarte JN, Avalos AM, Fang T, Barberena R, Sato S, Ploegh HL. Fluorophore-Conjugated Holliday Junctions for Generating Super-Bright Antibodies and Antibody Fragments. *Angew. Chem., Int. Ed.* 2015; 54:11706–11710.
38. Guimaraes CP, Witte MD, Theile CS, Bozkurt G, Kundrat L, Blom AE, Ploegh HL. Site-specific C-terminal and internal loop labeling of proteins using sortase-mediated reactions. *Nat. Protoc.* 2013; 8:1787–1799. [PubMed: 23989673]
39. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. Fiji: an open-source platform for biological-image analysis. *Nat. Methods.* 2012; 9:676–682. [PubMed: 22743772]
40. Brignull HR, Moore FE, Tang SJ, Morimoto RI. Polyglutamine proteins at the pathogenic threshold display neuron-specific aggregation in a pan-neuronal *Caenorhabditis elegans* model. *J. Neurosci.* 2006; 26:7597–7606. [PubMed: 16855087]

Figure 1.

Schematic representation of sortase reactions. Protein substrates equipped with a sortase A recognition sequence (LPXTG) can participate in (A) intermolecular transpeptidation reaction with small oligoglycine nucleophiles, (B) ligation reactions with other proteins containing a terminal oligoglycine portion, or (C) intramolecular transpeptidations to yield a circular adduct if exposure to the N-terminal glycine residue is given.

Figure 2.

SrtA_{7M} expression and lack of toxicity in *C. elegans*. (A,B) Longevity assays of animals expressing SrtA_{7M} as L3s or day 1 adults. L3 larvae (A) or day 1 adults (B) were heat-shocked (HS) for 30 min at 37 °C and thereafter incubated at 20 °C. Animals were scored in 2 day intervals. The *p*-values are >0.05 (Gehan–Breslow–Wilcoxon test). (C,D) motility assays of animals expressing SrtA_{7M}. Day 1 adults were heat-shocked for 30 min at 37 °C and thereafter incubated at 20 °C for 4 h (C) or 8 h (D) prior to testing. Number of body bends per 30 s was assessed. At least 30 animals were scored per condition.

Figure 3.

Sortase-mediated protein labeling. (A) A lysate of transgenic animals expressing GFP-LPETG and SrtA_{7m} following heat-shock was supplemented with G₃-biotin (1 mM) and 100 μL of sortagging reaction buffer (50 mM Tris, pH 7.5, 150 mM NaCl). (B) *In vivo* sortagging in *C. elegans*: animals expressing SrtA_{7m} upon heat-shocking and GFP-LPETG substrate were fed with G₃-biotin. The mixing control consists of a 1:1 mixture of lysates from animals either expressing GFP-LPETG substrate or SrtA_{7m} enzyme, previously fed with G₃-biotin (1 mM) and lysed in buffer supplemented with 1% SDS and 2% NP40. (C,D) *Phsp-16.2::srtA_{7m}; Pef-3::GFP-LPETG* strain was fed with G₃-Alexa 647 for 5 h at RT. Worms were transferred to a fresh plate for a further 24 h and (C) collected for imaging by confocal fluorescence microscopy or (D) lysed and resolved by SDS-polyacrylamide gel

electrophoresis (PAGE). Samples were visualized by immunoblotting (upper panel) or Typhoon in gel scanning (lower panel) at $\lambda_{\text{em}} = 647$ nm. The scale bar represents 10 μm . GFP-LPETG and SrtA_{7m} signal distributions shown in (C) are representative for the entire animal. Pearson colocalization coefficient for images shown in panel 4 is 0.754. All images were acquired with the same laser and exposure settings.

Figure 4.

GFP circularization by SrtA_{7m} in living nematodes. (A) Lanes 1 and 2: GFP circularization upon SrtA_{7m} expression: *Phsp-16.2::srtA; pef t-3::GFP* strains expressing circularizable G₅-GFP-LPETG substrate under the control of the *eft-3* promoter and SrtA_{7m}-HA controlled by a *hsp-16* promoter were lysed. The migration pattern of GFP was analyzed by SDS-PAGE, followed by immunoblotting. Lanes 3 and 4: Reversibility of GFP circularization in the presence of excess nucleophile was tested by adding an excess nucleophile (G₃-biotin) at 4 °C overnight to detergent-free lysates of induced (heat-shocked) or uninduced *Phsp-16.2::srtA_{7m}; Pef t-3::GFP* animals. Reversion of GFP circularization is characterized by the disappearance of the circular GFP signal observed in the immunoblot.

Figure 5.

SrtA_{7m} mediation of protein degradation in *C. elegans*. (A) Schematic illustration of ubiquitin-dependent degradation. SKP1-CUL1-Fbox protein ligase complexes (SCFs) mediate ubiquitin transfer from E2 enzymes to target substrates. The resulting ubiquitin-tagged substrates are degraded by the proteasome. Target specificity of the SCFs depends on substrate binding to F-box proteins (left panel). SrtA_{7m}-dependent covalent binding of substrates to F-box proteins enables degradation of an LPETG-tagged protein (right panel). (B) Immunoblot analysis of Ced10-LPETG-MYC substrate and G₃-Fbox-FLAG nucleophile levels in *Peft-3::Ced10, Phsp-16.2::Fbox* strain lysates. N2 strains were used as the control.

(C) Specific depletion of Ced10-LPETG-MYC following sortagging of the F-box to Ced10 in *Phsp-16.2::srtA_{7m}*; *Pef t-3::Ced10*; *phsp-16.2::Fbox* strains. N2 and *Phsp-16.2::srtA_{7m}* strains were used as controls. (D) Immunoblot analysis of different transgenic strains expressing combinations of Ced10-LPETG-MYC substrate, G₃-Fbox-flag nucleophile, and SrtA_{7m}. (E) Immunoblot analysis of Ced10-LPETG-MYC substrate degradation following SrtA_{7m}-mediated F-box ligation. *Phsp-16.2::srtA_{7m}*; *Pef t-3::Ced10* strains were heat-shocked for 90 min and thereafter kept at 20 °C. Samples were taken at the indicated time points. (F) Quantification of Ced10-LPETG-MYC substrate degradation shown in (E).

Table 1

Sortase, Substrate, and Nucleophile Constructs Used in This Study

protein	plasmid	promoter	forward primer	reverse primer
SrtA ^{7m}	pPD 49–100-HA-SrtA	hsp promoter	GAAGCTAGC/ATGCCGAAGCTGCTGTATCTTACGATGTGGCCTGA CTAC	GAACCAATGGTTATTCCAGGTTTAACCTCAGTGGCG
eGFP-LPETG	pPD 49–101-GFP-LPETG	eft-3 promoter	ATG AGT AAA GGA GAA GAA CTT TTC ACT GGA	TTATCCGGTCTCCGGGAGTTTGTAT
G5-GFP-LPETG	pPD 49–102-G5-GFP-LPETG	eft-3 promoter	CATTTTCAGGAGGACCCCTTGGCTAGC	TGATGACAGCCGGCCGATGCCGGAGCTCTTATTTGTATATGTTCAICCAATGCC
Ced10-LPETG-MYC	pPD 49–103-Ced10-LPETG-MYC	eft-3 promoter	CATTTTCAGGAGGACCCCTTGGCTAGCATGCAAGCGATCAAAATGTG	GATGACAGCGGCCCGATGCCGGAGCTCTTAGAGATCCTCTTTCGCTGA
G3-Fbox-FLAG	pPD 49–104-G3-Fbox-FLAG	hsp promoter	ATGTCTTCAC CCCACCGAGC	GATTTTGGATATAATTACTCTGTAGAAGACAT

Table 2

Antibodies Used in This Study

provider	specificity	dilution used
Santa Cruz Biotechnology	HA	1:20 000
Abcam	GFP	1:10 000
Cell Signaling	biotin	1:20 000
Santa Cruz Biotechnology	MYC	1:10 000
Abcam	FLAG	1:10 000