





## Sortase mediated protein ubiquitination with defined chain length and topology†

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Ubiquitination is a key post-translational modification on protein lysine sidechains known to impact protein stability, signal transduction cascades, protein–protein interactions, and beyond. Great strides have been made towards developing new methods to generate discrete chains of polyubiquitin and conjugate them onto proteins site-specifically, with methods ranging from chemical synthetic approaches, to enzymatic approaches and many in between. Previous work has demonstrated the utility of engineered variants of the bacterial transpeptidase enzyme sortase (SrtA) for conjugation of ubiquitin site-specifically onto target proteins. In this manuscript, we've combined the classical E1/E2-mediated polyubiquitin chain extension approach with sortase-mediated ligation and click chemistry to enable the generation of mono, di, and triubiquitinated proteins sfGFP and PCNA. We demonstrate the utility of this strategy to generate both K48-linked and K63-linked polyubiquitins and attach them both N-terminally and site-specifically to the proteins of interest. Further, we highlight differential activity between two commonly employed sortase variants, SrtA 5M and 7M, and demonstrate that while SrtA 7M can be used to conjugate these ubiquitins to substrates, SrtA 5M can be employed to release the ubiquitin from the substrates as well as to cleave C-terminal tags from the ubiquitin variants used. Overall, we envision that this approach is broadly applicable to readily generate discrete polyubiquitin chains of any linkage type that is accessible via E1/E2 systems and conjugate site-specifically onto proteins of interest, thus granting access to bespoke ubiquitinated proteins that are not currently possible.

## Introduction

The conjugation of ubiquitin, a 76-amino acid protein, to protein sidechains is one of the most common protein post-translational modifications (PTMs) with over 63 000 sites

reported across the proteome.<sup>1</sup> The process of ubiquitination (also commonly referred to as ubiquitylation), where the ε-amino group on a lysine (K) sidechain forms an isopeptide linkage with the C-terminal glycine of ubiquitin, is enacted by a cascade of E1 activating enzymes, E2 conjugating enzymes, and E3 ligase enzymes, and is removed by a class of proteases called deubiquitinating enzymes (DUBs).<sup>2</sup> Ubiquitin itself can act as a substrate *via* one of its own seven lysines to generate polyubiquitin chains, which have varying topologies and in turn biological roles in the cell. For example, K48-linked polyubiquitin is the canonical signal for proteasomal degradation of proteins,<sup>3</sup> K63-linked polyubiquitin facilitates the cellular DNA damage response,<sup>4</sup> and some atypical chains (such as K6-linked polyubiquitin) have biological roles that are not yet fully understood.<sup>5</sup> While many strides have been made in the field towards the generation of ubiquitinated substrates with enzymatic,<sup>6</sup> fully-synthetic,<sup>7,8</sup> and various semi-synthetic methods<sup>9,10</sup> available, there are still challenges to generating site specific, custom polyubiquitinated proteins in a robust, high yielding, and modular manner.

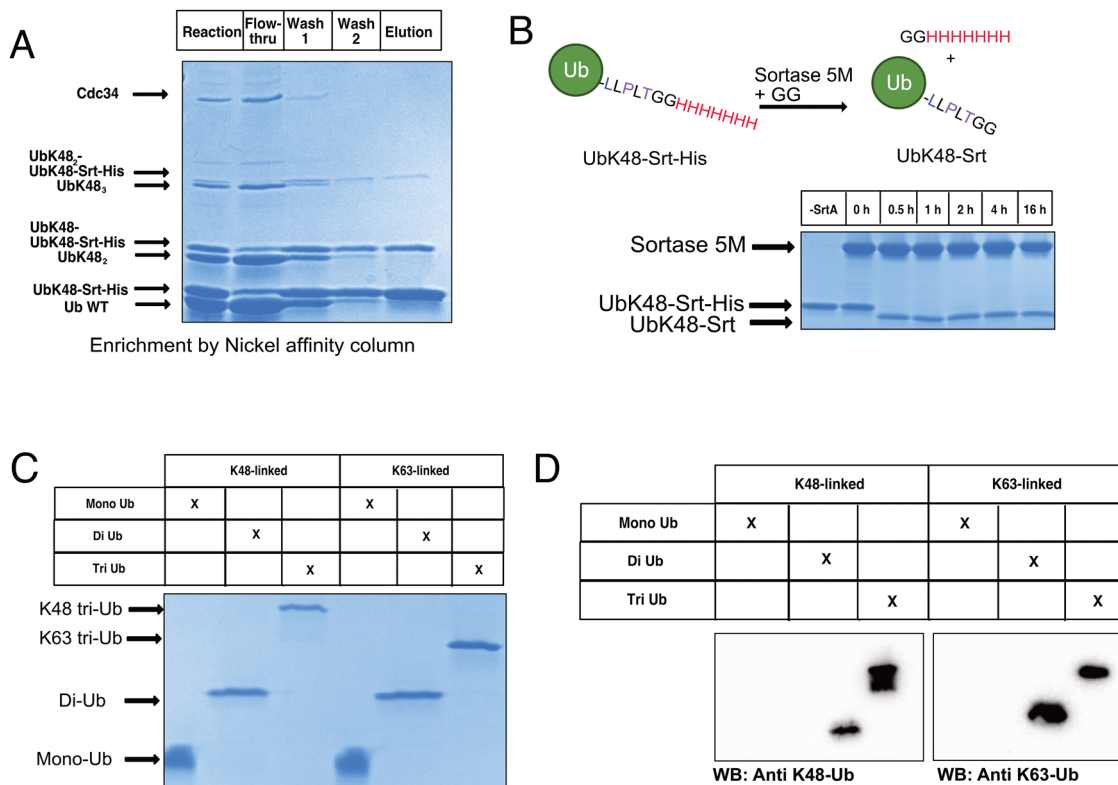
In addition to the challenges that exist for the robust generation of custom polyubiquitin chains, there are limitations to the current strategies for the site-specific attachment of polyubiquitin chains onto proteins of interest. Commonly employed approaches utilize chemistry-driven methods such as native chemical ligation (NCL)<sup>8,11,12</sup> and thiol-alkene conjugation<sup>13,14</sup> and endopeptidase strategies.<sup>15</sup> Recently, the Lang group demonstrated a methodology to site-specifically ubiquitinate proteins utilizing the bacterial transpeptidase enzyme sortase A (SrtA) from *Staphylococcus aureus*.<sup>16</sup> Sortase was first discovered as a key enzyme involved in bacterial cell wall assembly and has been posited to be a potential target for antimicrobial therapies,<sup>17,18</sup> but has since also been employed in countless bioconjugation applications including self-cleaving peptides and proteins,<sup>19,20</sup> antibody–drug conjugate assembly,<sup>21</sup> and many more.<sup>22</sup> Broadly, sortase utilizes its own catalytic cysteine residue to catalyze the transfer of a peptide bearing an LPXTG motif onto an N-terminal glycine-bearing peptide substrate (Scheme 1). Sortase A from *Staphylococcus aureus* in particular

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**Fig. 1** Generating sortase compatible polyubiquitin. (A) SDS-PAGE gel stained with Coomassie Blue showing IMAC purification of His-tagged polyubiquitin chains. (B) SrtA 5M-mediated cleavage of the C-terminal His-tag on UbKXSrt. (C) SDS-PAGE gel stained with Coomassie showing purification of mono, di, and triubiquitin bearing C-terminal sortase recognition motif post-cation exchange chromatography. (D) Western blots of di and triubiquitin using linkage-specific antibodies (K48-specific on left and K63-specific on right).

### Generating N-terminally ubiquitinated GFP

Having access to multiple sortase compatible ubiquitin chains, we sought to test the sortase mediated conjugation to a model protein with an N-terminal glycine. For this application, we cloned a superfolder green fluorescent protein bearing a V1G mutation (sfGFP-V1G) to use as the acceptor substrate. We then utilized lysine-free ubiquitin variant UbK0Srt and performed conjugation reactions at 37 °C with SrtA 7M and observed formation of the monoubiquitinated sfGFP (UbK0Srt-sfGFP) species in as little as 30 minutes, with the highest amount of UbK0Srt-sfGFP present at 16 hours (Fig. 2A and B).

Following the generation of UbK0Srt-sfGFP, we were curious about the reversibility of this reaction. After reaction of SrtA 5M with UbK0Srt-sfGFP, we observed slower hydrolysis of UbK0Srt-sfGFP than the hydrolysis of the His-tag from UbK48Srt-His (Fig. 1B), with gradual hydrolysis leading to near-complete cleavage of UbK0Srt-sfGFP after 16 hours (Fig. 2C and D). When the cleavage assays were conducted in the presence of 1 mM GG and GGG peptides, we observed a faster rate of cleavage (estimated half-life ~2 h) by SrtA 5M for both sets of GG/GGG peptides (Fig. S3, ESI<sup>†</sup>), while we observed no cleavage of the UbK0Srt-sfGFP species after 16 h when using SrtA 7M (Fig. S2, ESI<sup>†</sup>). Further, when cleaving with SrtA 5M in the presence of GG peptide, we observed near-quantitative production of the original UbK0Srt species by LC/MS (Fig. S1, ESI<sup>†</sup>). This provided

useful insight into the use of the two sortase variants for control of our experimental system.

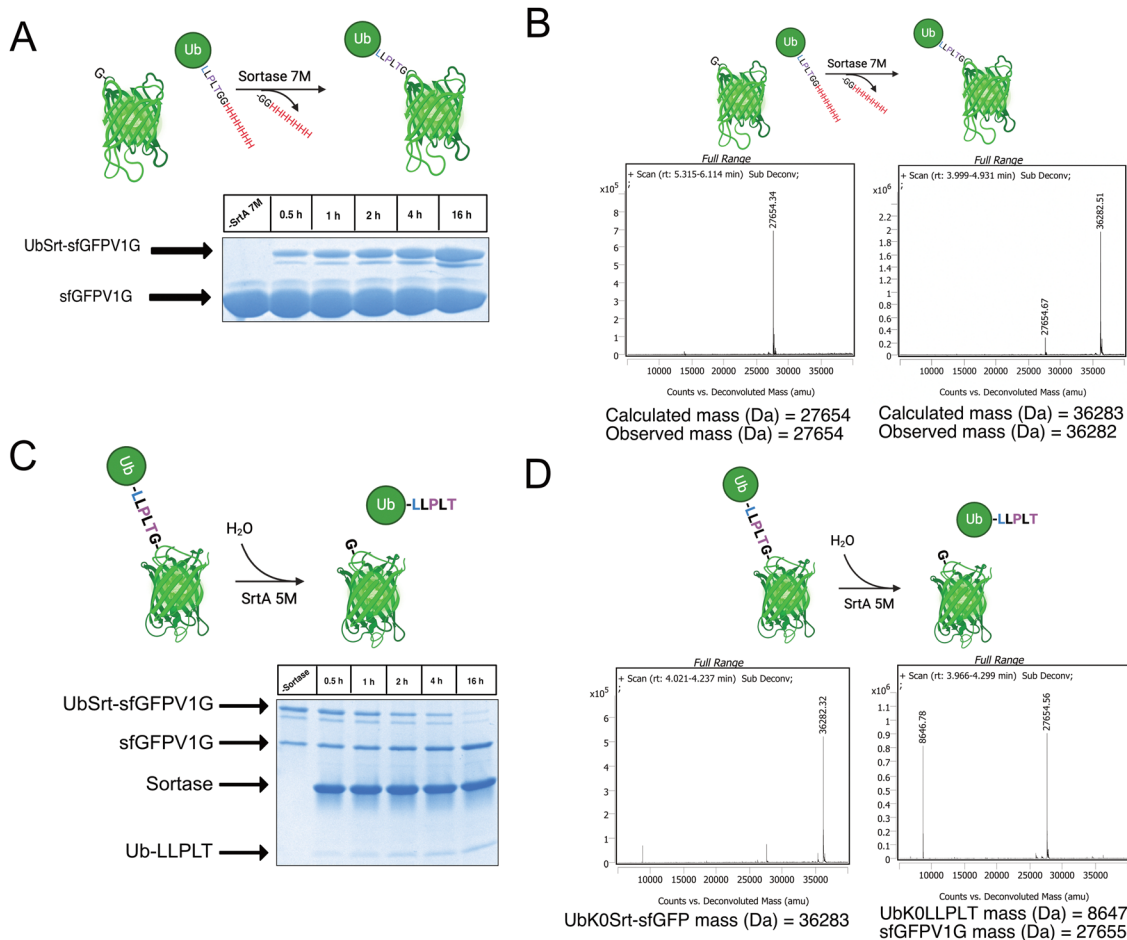
### Generating internally polyubiquitinated sfGFP

Since most ubiquitination events occur at internal lysines, we sought to use our approach to ubiquitinate an internal position of sfGFP. By using genetic code expansion (GCE) to site-specifically install *para*-Azido Phenylalanine (*pAzF*)<sup>30</sup> into sfGFP (Fig. 3B, left), we were able to then perform strain-promoted azide-alkyne cycloaddition (SPAAC)<sup>31</sup> with Gly-Gly-Gly-Dibenzocyclooctyne (GGG-DBCO) (Fig. 3B, right) to provide a sortase substrate as a defined internal position. Our initial trial with sfGFP-150*pAzF*-DBCO-GGG demonstrated that SrtA 7M could readily deposit UbK48Srt onto the internal tri-glycyl site (Fig. 3C). We then combined our sortase compatible ubiquitin chains with defined length and topology (Fig. 1C and D) with our SPAAC approach to yield internally ubiquitinated proteins bearing the desired chain type and length (Fig. 3C, D and Fig. S6, ESI<sup>†</sup>).

### Generating polyubiquitinated PCNA

To further exemplify the broader uses of this approach, we tested this workflow on DNA replication protein PCNA. PCNA is a protein which trimerizes to form a “clamp” around the DNA, and thus is a key protein in DNA replication as its classic function assists in recruiting and maintaining attachment of





**Fig. 2** Generation of N-terminally mono and polyubiquitinated sfGFP. (A) Schematic and SDS-PAGE gel stained with Coomassie Blue showing SrtA 7M-mediated ubiquitination of sfGFPV1G over 0.5–16 hours. (B) Deconvoluted intact mass spectrometry data demonstrating N-terminal attachment of UbK0Srt to sfGFPV1G. (C) SDS-PAGE gel stained with Coomassie showing SrtA 5M-mediated hydrolysis of UbK0Srt-sfGFPV1G. (D) Deconvoluted QTOF-MS data demonstrating SrtA 5M-mediated hydrolysis of UbK0Srt-sfGFPV1G to UbK0LLPLT and sfGFPV1G.

the DNA polymerase to the template DNA.<sup>32</sup> PCNA serves critical roles in maintaining genomic integrity in cells; if the replication machinery encounters DNA damage lesions, PCNA is ubiquitinated in response and facilitates either “error-free” homologous template switching or error-prone translesion synthesis. The decision between template switching or translesion synthesis is dependent on the ubiquitination of PCNA; the former being enabled by K63-linked polyubiquitination, and the latter being enabled by mono-ubiquitination of PCNA.<sup>27</sup> PCNA has been reported to have additional possible modes of ubiquitination,<sup>33,34</sup> but these are understudied due to a lack of tools.

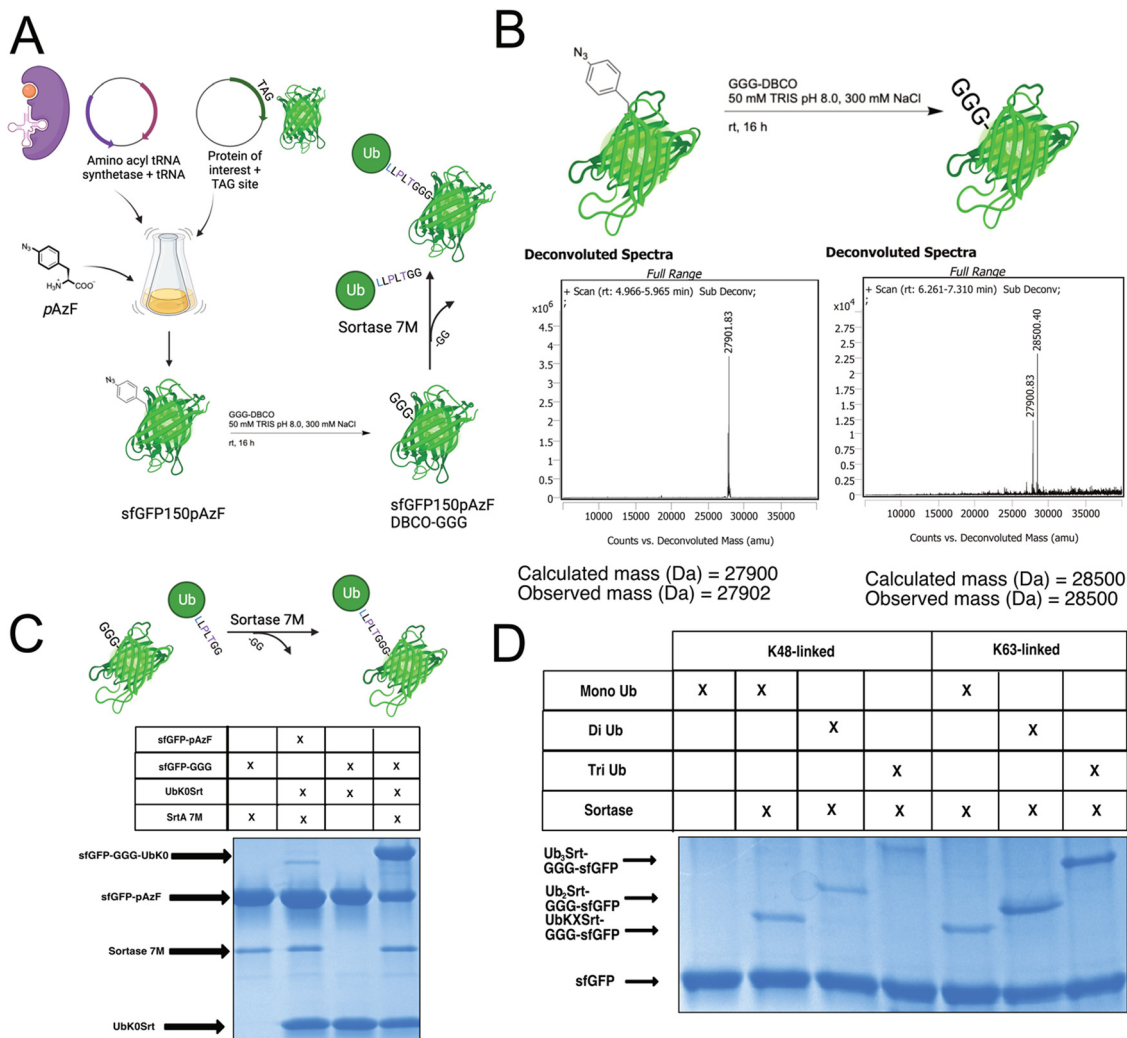
To generate site-specifically (poly)ubiquitinated PCNA, we first performed GCE to incorporate the *pAzF* click handle into the protein (Fig. 4A and B, left). We selected K164 as the point of conjugation given its significance in DNA translesion synthesis. To do so, we mutated the K164 position to an Amber stop codon (TAG) for GCE and added a C-terminal His-tag for purification. *pAzF* incorporation was robust at this site, with the final PCNA product showing high fidelity incorporation of the *pAzF* (Fig. 4B, left and Fig. S5, ESI<sup>†</sup>). Following purification,

the PCNA-164*pAzF* was subjected to SPAAC with GGG-DBCO to install the oligoglycine handle for sortylation (Fig. 4B). Following SPAAC, we utilized SrtA 7M to conjugate purified mono (Fig. 4C), di, and triubiquitin linked by either K48 or K63 to the PCNA trimers at site 164. Both K48-linked and K63-linked polyubiquitin were successfully conjugated onto this position and were visible by SDS-PAGE stained with Coomassie Blue (Fig. 4D) as well as Western blotting against ubiquitin, K48-linked polyubiquitin, or K63-linked polyubiquitin (Fig. 4E and Fig. S7, ESI<sup>†</sup>) thus demonstrating our approach.

Given pre-existing work in the field (outlined in ref. 16 and 19), we wanted to highlight some key features of our technology compared to what is currently published. The Lang group has previously developed and reported a system for GCE which incorporates a novel unnatural amino acid, azidoglycylglycyl lysine (AzGGK), which enables the use of sortase mediated ligation directly onto lysine residues at the position of interest on a protein. This generates an isopeptide linkage that is more akin to a native ubiquitination, which may be desirable in instances where a near-native linkage is critical to the experimental system it is being applied to. The incorporation







**Fig. 3** Generating internally mono and polyubiquitinated sfGFP *via* click chemistry and sortase. (A) Schematic of workflow for genetic code expansion, click chemistry, and ubiquitination *via* sortase. (B) Reaction conditions and deconvoluted intact protein mass spec showing successful GGG-DBCO attachment to sfGFP150pAzF. (C) SDS-PAGE gel stained with Coomassie showing site-specific monoubiquitination of sfGFPpAzF150-DBCO-GGG *via* sortylation (approx. 60% conversion based on densitometry). (D) SDS-PAGE gel stained with Coomassie Blue showing site specific attachment of K48 and K63-linked mono, di, and tri ubiquitin onto sfGFP-150pAzF-DBCO-GGG.

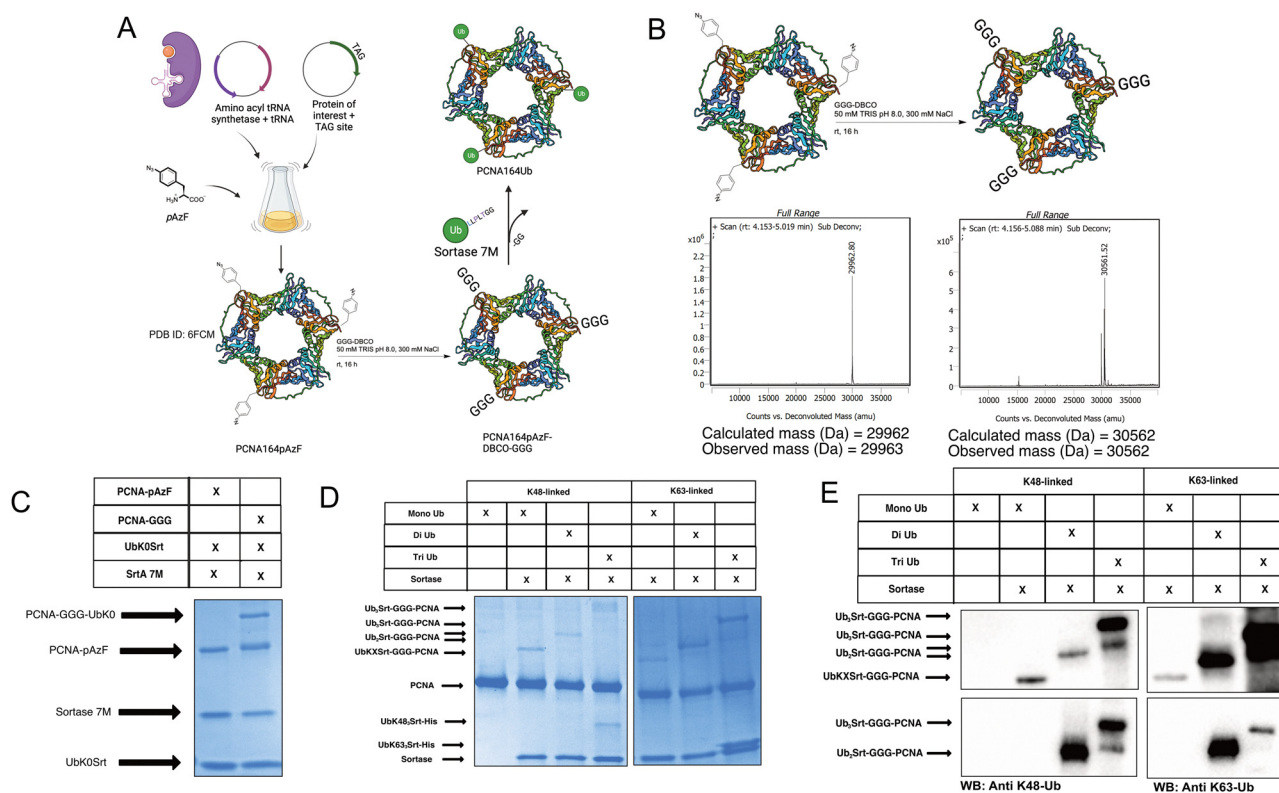
of pAzF in our hands is very robust and while the click chemistry results in a less native linkage between the ubiquitin and protein of interest, the use of commercially available pAzF and GGG-DBCO abrogate the need for chemical synthesis of the AzGGK, making this strategy more accessible to biochemistry labs and to those who may want to avoid chemical synthesis of reagents. The Lang group has also used a derivation of this technology to generate polyubiquitin chains by generating ubiquitin dimers *via* and E1/E2 system and using the AzGGK/sortase approach to make longer ubiquitin chains by incorporating the AzGGK into the ubiquitin monomers themselves. While this system presents a robust approach to generate polyubiquitin chains, our strategy which utilizes wild-type ubiquitin leaves opportunity for GCE to be used in alternative ways (*e.g.* incorporating click or photoaffinity handles, post-translational modifications such as phosphorylation) throughout the ubiquitin chains without the complication of introducing multiple GCE sites into

the ubiquitin monomers. Finally, through our blended approach using SrtA 7M for forward conjugation and SrtA 5M in the presence of diglycine for cleavage, we can recover unspent ubiquitin from the reactions by purification since the system disfavors hydrolysis of the sortase recognition motif in unconjugated ubiquitin monomers.

## Conclusions

Here we present a method for the facile generation and conjugation of defined ubiquitin chains to proteins of interest, thus providing a new technology to biochemically test hypotheses surrounding ubiquitination events. In doing so, we demonstrate the utility of a transient C-terminal His-tag to facilitate enrichment and purification of sortase compatible ubiquitin chains and the application of GCE and SPAAC to





**Fig. 4** Generating site-specifically polyubiquitinated PCNA. (A) Schematic showing general workflow of generation of PCNA-164Ub<sub>n</sub>. [PDB ID: 6FCM]<sup>35</sup> (B) Schematic and deconvoluted intact-protein mass spec showing pAzF incorporation and GGG-DBCO click chemistry. (C) SDS-PAGE gel stained with Coomassie showing click-dependent monoubiquitination of PCNA164pAzF. (approx. 60% conversion based on densitometry). (D) SDS-PAGE gel stained with Coomassie showing mono, di, and triubiquitination of PCNA with K48-linked and K63-linked polyubiquitin. (E) Western blots showing successful conjugation of polyubiquitins to PCNA164pAzF-DBCO-GGG. Immunoblot against ubiquitin (top), K48-linked polyubiquitin (bottom left), and K63-linked polyubiquitin (bottom right).

conjugate bespoke chains to sites of interest. Additionally, we have demonstrated and expanded upon differing activities which have been previously noted<sup>36</sup> between the SrtA 5M mutants and the 7M mutants and leveraged their differential activities to our benefit.

Taken together, the work presented here expands on the current knowledge, use, and applications of sortase technology, as well as providing valuable methods for generating ubiquitinated protein substrates *via* sortase-mediated ligation. This suite of reagents and methodologies provides access to ubiquitinated proteins with greater control and homogeneity than previous approaches, which will enable new avenues for the exploration of the ubiquitin code and enable us, and others, to explore the ubiquitin code more extensively.

## Author contributions

GMB: conceptualization, funding acquisition, investigation, methodology, resources, supervision, writing original draft, writing – review and editing. NRR: investigation, methodology, resources, writing original draft, writing – review and editing. JNB: investigation, resources, writing – review and editing. MIN: resources, writing – review and editing.

## Conflicts of interest

There are no conflicts to declare.

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