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Review

Sortases: structure, mechanism, and implications for protein engineering

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Sortase enzymes are critical cysteine transpeptidases on the surface of bacteria that attach proteins to the cell wall and are involved in the construction of bacterial pili. Due to their ability to recognize specific substrates and covalently ligate a range of reaction partners, sortases are widely used in protein engineering applications via sortase-mediated ligation (SML) strategies. In this review, we discuss recent structural studies elucidating key aspects of sortase specificity and the catalytic mechanism. We also highlight select recent applications of SML, including examples where fundamental studies of sortase structure and function have informed the continued development of these enzymes as tools for protein engineering.

Highlights

Sortases are a widespread family of bacterial transpeptidases that covalently anchor proteins to the cell surface and assemble bacterial pili.

The structure and mechanism of sortases remain important topics of current research, and recent structure–function studies have revealed key aspects of sortase A substrate recognition, with important mechanistic questions remaining to be answered.

Sortases continue to see widespread use in protein engineering as part of sortase-mediated ligation (SML) strategies; advances are supported by studies on specificity, structure, and engineering of novel variants.

Significant opportunities remain for the continued development of SML, including expanding substrate scope and utilizing enzymes other than *Staphylococcus aureus* sortase A and related mutants.

Bacterial sortases: cell wall functionalization and protein engineering

Sortase enzymes are membrane-bound proteins located on the surface of **Gram-positive** (see Glossary) bacteria, and are also present to a limited extent in Gram-negative and archaebacteria [1]. First discovered in *Staphylococcus aureus* in 1999 by Mazmanian *et al.* [2,3], the sortase family has expanded to six classes (A–F) with thousands of members among Gram-positive bacteria, although not all species contain sortases from each class [4,5]. The biological role of these enzymes is functionalization of the bacterial cell surface. Sortases achieve this by catalyzing transpeptidation reactions in which substrate proteins displaying specific target sequences (e.g., LPXTG) are covalently ligated to cell wall precursors or linked together to form the bacterial pilus [6].

As a specific example of cell wall anchoring, sortase A from *S. aureus* (saSrtA) is responsible for linking a range of substrates to the peptidoglycan, including factors that promote host colonization and bacterial pathogenesis [7]. SaSrtA (anchored via an N-terminal transmembrane segment) recognizes substrates displaying a C-terminal **cell wall sorting signal (CWSS)** [2,8–10] (Figure 1A). This CWSS consists of the requisite LPXTG motif, followed by a transmembrane segment and a short tail of positively charged residues. The active site cysteine of saSrtA cleaves between the P1 threonine and P1′ glycine residues, generating a thioester-linked acyl enzyme intermediate. This is then resolved through nucleophilic attack by the amine terminus of the interpeptide bridge (pentaglycine) of the cell wall precursor **lipid II**. The substrate protein, now linked to lipid II through an amide bond, is then incorporated into the developing peptidoglycan through a series of subsequent transpeptidation and transglycosylation reactions [1]. Moving beyond *S. aureus*, a similar mechanism is expected for other sortases, however the five-residue substrate motif and identity of the amino nucleophile can vary between organisms and different sortase classes [1]. In addition, sortase-mediated construction of the bacterial pilus involves additional mechanistic steps, most notably isopeptide bond formation to covalently link the subunits of pili prior to cell wall incorporation [1].

As noted above, sortases decorate the bacterial cell surface with a range of factors that promote bacterial virulence [7,11]. Therefore, these enzymes have attracted attention as targets for novel

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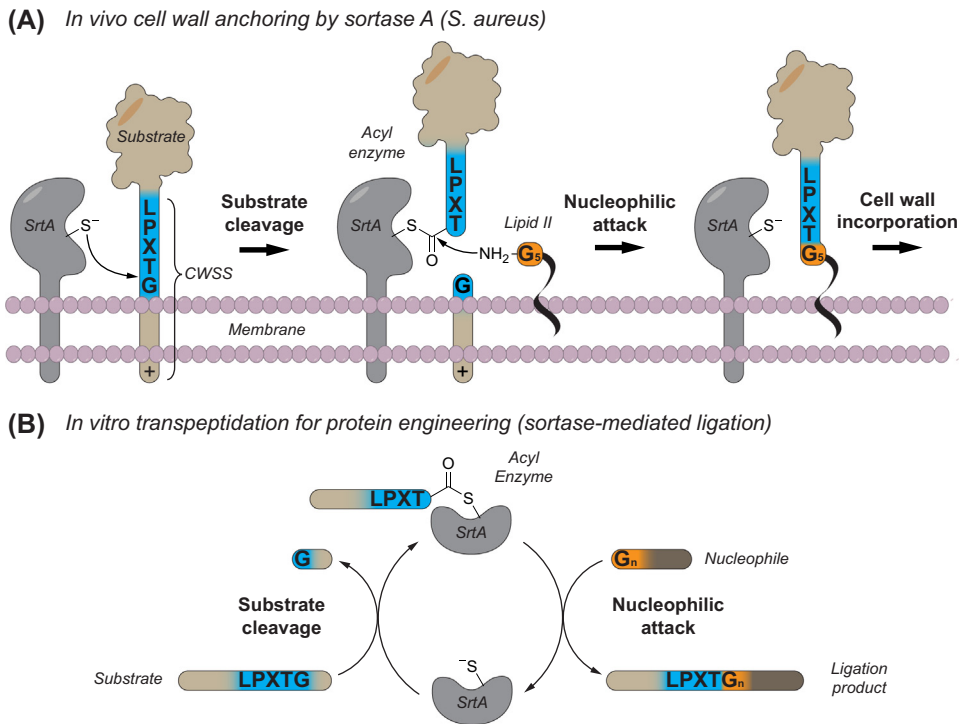

Figure 1. Cell wall functionalization and protein engineering mediated by class A sortase (SrtA). (A) In the currently accepted mechanism for cell wall anchoring (depicted here for *Staphylococcus aureus*), SrtA recognizes substrates containing a cell wall sorting signal (CWSS). This CWSS includes the SrtA recognition motif (LPXTG), a transmembrane segment and short positively charged tail (+). SrtA cleaves between the threonine (T) and glycine (G) residues to generate an acyl-enyzme intermediate. This then reacts with the N terminus of the interpeptide bridge of lipid II (in the case of *S. aureus* this is pentaglycine, G_5). The lipid II conjugated protein is then incorporated into the bacterial cell wall. (B) *In vitro* transpeptidation using soluble SrtA enzymes allows for covalent ligation of reaction partners in a process termed sortase-mediated ligation (SML). In its most common form, this involves LPXTG-containing substrates and nucleophiles containing one or N-terminal glycine residues. The SML technique has emerged as a powerful method for protein engineering.

antibiotic development. Indeed, this is an ongoing area of significant interest and the reader is referred to a number of reviews focused on sortase inhibitor development [11–13]. While the potential to hinder infection of Gram-positive pathogens (e.g., methicillin-resistant *S. aureus* or *Streptococcus pneumoniae*) is of widespread importance, perhaps of even higher significance is the use of sortases in **sortase-mediated ligation (SML)** over the past ~20 years as a powerful new tool in protein engineering. The SML approach exploits the transpeptidation activity of sortase enzymes to covalently link a range of reaction partners through a relatively small ligation site (e.g. $LPXTG_n$, n = 1–5) (Figure 1B). The potential of this technique was first demonstrated in 2004, where the soluble catalytic domain of saSrtA was used to successfully catalyze *in vitro* transpeptidation reactions using both peptide- and GFP-based substrates [14]. Subsequent years have seen numerous and varied examples of SML, increasingly using engineered variants of *S. aureus* SrtA (saSrtA), as well as *Streptococcus pyogenes* SrtA (spySrtA), the pilus-building SrtC from *Corynebacterium diphtheriae*, and others [15]. The scope of applications relying on SML has also grown significantly, and includes work in the areas of biomaterials, molecular imaging, cell surface labeling, targeted therapeutics, vaccine development and others [16–20].

In this review, we have elected to focus on a subset of work from the last ~25 years of sortase research. First, we discuss how recent structural studies of class A sortases have informed our

Glossary

Antibody– or nanobody–drug conjugates (ADC or NDCs): antibodies are proteins produced by the immune system that recognize and bind to foreign molecules or antigens. The most common type of antibody is IgG, which consists of four polypeptide chains connected by disulfide bonds and two heavy and two light chains. ADCs involve the covalent attachment of therapeutics, typically cytotoxic drugs, to antibodies. NDCs are based on nanobodies, which are single-domain antibodies derived from camelids.

Cell wall sorting signal (CWSS): a sequence motif found in sortase substrates that leads to the anchoring of these proteins to the bacterial cell wall or assembly into pili. For class A sortases, the CWSS consists of a C-terminal Leu–Pro–X–Thr–Gly (or LPXTG, where X=any amino acid) motif, followed by a hydrophobic transmembrane segment and a short positively charged tail.

Gram-positive: bacteria are often characterized as being either Gram-positive or Gram-negative, based on the results of a Gram stain test that differentiates between the two types of cell walls. Gram-positive bacteria generally contain a single outer membrane with a thick peptidoglycan layer, consisting of sugars and amino acids. In contrast, Gram-negative bacteria contain a relatively thin peptidoglycan layer which is located between outer and inner cell membranes.

Lipid II: a precursor molecule of the bacterial cell wall that serves as the ligation partner for many sortase substrates *in vivo*. Subsequent incorporation of the lipid-II-modified protein into the peptidoglycan serves to anchor the protein to the bacterial cell wall.

Sortase-mediated ligation (SML): in biochemistry, ligation refers to the joining together of biomolecules, for example, nucleic acids, polysaccharides, or polypeptides, with a new chemical bond. SML utilizes the transpeptidation reaction catalyzed by bacterial sortases to combine two fragments via a new peptide (or isopeptide) bond.

understanding of how substrates are bound and accommodated in the enzyme active site. This emphasis on sortase A enzymes is motivated by their central role in protein engineering (SML), and well as the status of sortase A enzymes as the founding members of the sortase enzyme family. We then discuss the implications of these structural studies for the sortase catalytic mechanism. Finally, we conclude with a brief overview of developments in the SML field, including examples that have been inspired by ongoing fundamental work on sortase structure and function.

Structure and mechanism of class A sortases

Sortase A structures

The first structure of saSrtA was determined in 2001, using NMR [9]. Notably, the protein construct used here, and for most sortase applications, included only the soluble catalytic domain of the enzyme, excluding the N-terminal transmembrane region. For saSrtA, this construct started at Q60 (using UniProt ID SRTA_STAA8). This structure revealed a core eight-stranded antiparallel β-barrel, which is conserved across the sortase superfamily and is referred to as the sortase fold (Figure 2A,B) [21,22]. This structure also revealed the presence of a highly conserved cysteine (C184) and histidine (H120) in the putative enzyme active site; both of which were shown in other studies to be required for saSrtA activity [8,23]. These residues, along with a conserved arginine (R197), are often considered the core catalytic triad (Cys–His–Arg) of sortase A enzymes [1]. This NMR study also began to elucidate the binding site for Ca^{2+}, which is necessary for maximum saSrtA activity. Subsequent NMR studies determined that Ca^{2+} binding is mediated by a series of acidic residues (E105, E108, D112, and E171) and N114, which in turn reduces the mobility of a loop (β6–β7 loop) flanking the saSrtA active site and likely enhances recognition of the LPXTG substrate [24].

The next structures of saSrtA, reported in 2004 using X-ray crystallography, included the apo enzyme [Protein Data Bank (PDB) ID 1T2P] and a catalytically inactive mutant (C184A) noncovalently bound to an LPETG peptide (PDB ID 1T2W) [21]. Neither of these structures contained calcium, suggesting they do not represent fully active conformations of saSrtA. In 1T2W, the LPETG peptide was significantly shifted in the presumed substrate-binding pocket. Specifically, a distance of 5.6 Å was observed between the C184A methyl side chain and P1 Thr carbonyl carbon, where initial nucleophilic attack occurs [21]. It is therefore likely that this represents a catalytically incompetent binding interaction.

Subsequent peptide-bound NMR structures of class A sortases included synthetic complexes of saSrtA (PDB ID 2KID) and *Bacillus anthracis* SrtA (baSrtA, PDB ID 2RUI) with a peptidomimetic, LPAT* where T* = (2*R*,3*S*)-3-amino-4-mercapto-2-butanol, replacing the P1 Thr carbonyl with a thiol that allows for disulfide bond formation with the catalytic cysteine of each enzyme [25,26]. Here, the structure of saSrtA is bound to calcium and likely represents the active conformation of the enzyme. These structures, in combination with additional NMR experiments, nicely support intermolecular interactions in the S4 and S3 binding sites that contact the P4 Leu and P3 Pro residues of the LPXTG motif [25]. However, because of the nature of the T* residue, the P1 Thr carbonyl is absent, which precludes observation of all relevant interactions that lead to substrate cleavage and formation of the acyl enzyme intermediate. The saSrtA-LPAT* (PDB ID 2KID) and baSrtA-LPAT* (PDB ID 2RUI) structures also suggested heterogeneity in the way that the P1 Thr residue is accommodated in the enzyme active site. Specifically, in the saSrtA structure, the P1 Thr sidechain was observed to project out into solvent, in a conformation that has been termed 'threonine-out' (Thr-out) [26]. In contrast, the corresponding P1 Thr in the baSrtA complex projected into the substrate binding cleft, adopting a 'threonine-in' (Thr-in) conformation [26]. Rather than representing fundamentally distinct modes of LPXTG recognition by baSrtA and saSrtA, MD simulations have suggested that the LPXT fragment in the acyl enyzme

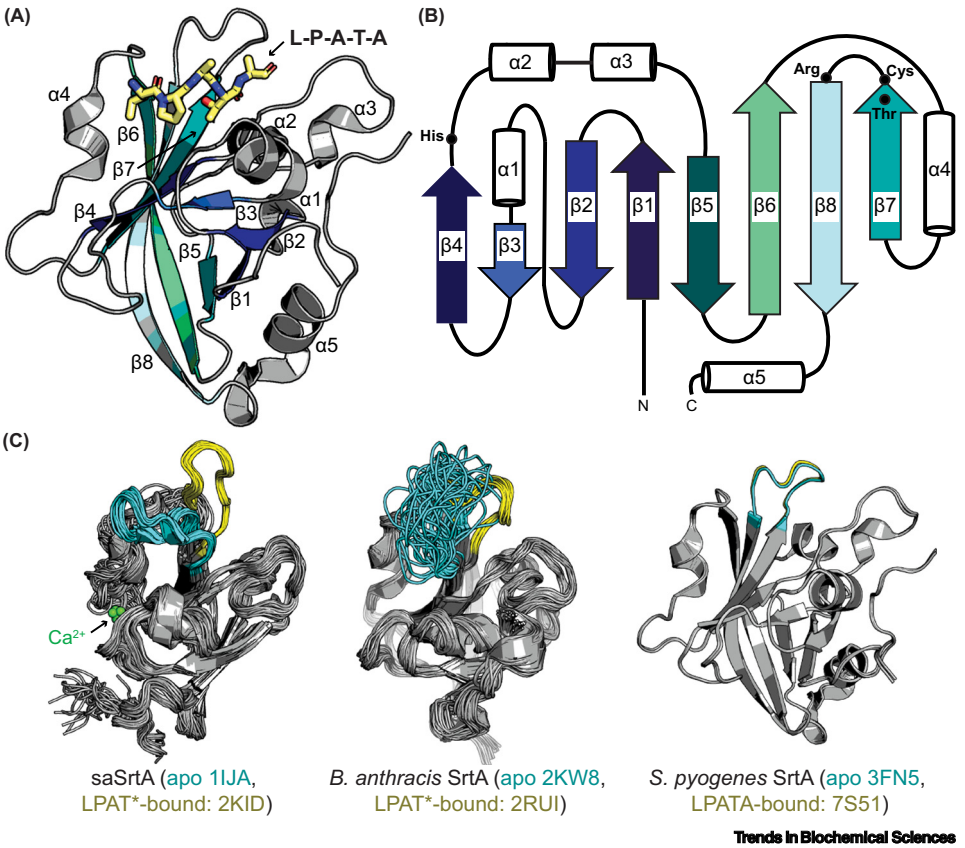

Figure 2. Sortase A (SrtA) structure and substrate binding dynamics. (A) The crystal structure of *Streptococcus pyogenes* SrtA bound to the LPATA peptide substrate (PDB IDs 7S51, [28]) is shown in cartoon representation and colored by secondary structure element, specifically, the β-strands (β1–β8) that characterize the sortase fold. The peptide is shown as sticks and colored by atom (C = yellow, O = red, N = blue). All SrtA proteins share these structural features, including the α-helices labeled α1–α5. Sortases from other families (B–F) contain the β-sheets highlighted, but the number of α-helices vary per class. (B) A 2D visual representation of the structure in (A). Residues important for catalysis, including the traditional catalytic His–Cys–Arg triad, as well as Thr discussed in this Review, are highlighted. (C) Differing β7–β8 loop dynamics may play a role in SrtA activity and/or specificity. NMR structures of saSrtA (left, PDB IDs 1IJA, and 2KID, [25,26]) and *Bacillus anthracis* SrtA (middle, 2KW8 and 2RUI) show changes in loop conformation upon ligand binding, including a conformational change (saSrtA) or disordered-to-ordered transition (*B. anthracis* SrtA). In contrast, *Streptococcus pyogenes* SrtA (right, 3FN5 and 7S51, [28,43]) reveals almost identical loop states in the presence or absence of bound peptide. All structures are in gray carton representation, with loops colored as labeled. Bound calcium atoms in the saSrtA active conformation (2KID) are in green, as highlighted.

intermediate of saSrtA readily samples both Thr-in and Thr-out states, with the Thr-in conformation likely to be the most relevant for catalysis [27]. The Thr-in conformation has also been suggested to facilitate contacts with the conserved arginine of the Cys-His-Arg triad characteristic of sortase A enzymes (see below) [1,26].

More recently, we successfully crystallized and solved the structures of spySrtA bound to the target sequences LPATA and LPATS (PDB IDs 7S4O and 7S51, respectively), as well as a peptidomimetic of the ligation product, LPAT–lipid II, in two distinct conformations, P1 Thr-in and Thr-out (PDB IDs 7T8Y and 7T8Z, respectively) [28]. For all these structures, a catalytically inactive spySrtA mutant (C208A) was utilized. These structures revealed key similarities with the previous LPAT*-bound saSrtA and *B. anthracis* SrtA structures, in particular regarding the

positioning and recognition of the P4 Leu and P3 Pro positions [25,26,28]. We also see Thr-in versus Thr-out switching as noted above, suggesting that both conformations are available to spySrtA as has been suggested for saSrtA [27].

Moving to other residues in the pentapeptide substrate motif, our recent spySrtA structures are notable in that they contain full LPATX (X = S or A) sequences. This provided initial insight into interactions at the P1′ substrate position for spySrtA, primarily in that no specific intermolecular contacts were observed. This is consistent with recent *in vitro* work demonstrating elevated substrate promiscuity at the P1′ site for spySrtA and related streptococcal SrtA enzymes [22,29,30]. This substrate promiscuity stands in sharp contrast to the stringent P1′ Gly specificity of saSrtA [31], and at present available structural data for SrtA enzymes does not provide a clear explanation for this difference in substrate scope. We currently hypothesize that P1′ specificity is controlled by flexibility in the β7–β8 loop, a region whose sequence and length varies considerably amongst class A sortases [4]. Where comparisons are available, there appears to be a wide spectrum of loop dynamics in this region; for example, while the apo and peptide-bound structures of *S. pyogenes* SrtA show minimal loop differences (PDB ID 3FN5 versus 7S51), saSrtA reveals a major conformational change (PDB ID 1IJA/1T2P versus 2KID), and *B. anthracis* SrtA undergoes a disordered-to-ordered transition upon substrate binding (PDB ID 2KW8 versus 2RUI) (Figure 2C) [9,25,26,28]. Beyond these observations, a key role for the β7–β8 loop in P1′ specificity is also supported by experimental work showing that chimeric SrtA enzymes containing different β7–β8 loop residues exhibit variations in their tolerance of residues at the P1′ position [22,29,32].

Building on the substrate-bound structures of spySrtA, an additional crystal structure of this enzyme bound to a noncanonical substrate motif (LPALA) was reported by us in early 2024 [33]. Substrates of this type (e.g., LPKLG) were previously shown to readily react with spySrtA and related streptococcal enzymes [34], suggesting that they bound in a fashion similar to the more standard LPATX (X = S or A) described above. Indeed, the solved crystal structure showed that the LPALA bound in a similar fashion to LPATA, with the P1′ Leu occupying the same binding site as the canonical P1′ Thr in a Thr-in conformation [33].

In summary, a significant amount of structural work has been reported for sortase A enzymes, leading to key insights into the way these enzymes recognize their substrates. Currently, there are 80 sortase structures in the PDB, 31 of which are of class A sortases, from *S. aureus* and *B. anthracis*, as well as *Streptococcus agalactiae*, *S. pneumoniae*, *S. pyogenes*, *Actinomyces oris*, *Lactobacillus rhamnosus*, *Listeria monocytogenes*, and *Streptococcus mutans* (Table 1) [9,21,25,26,28,29,35–44]. While the sortase fold is conserved amongst these, the *S. pneumoniae* structures (PDB IDs 4O8L, 4O8T, and 5DV0) are all of a domain-swapped dimer, which is likely catalytically inactive [30]. In addition, the first *S. agalactiae* SrtA structure deposited (PBD ID 3RCC) was truncated at the C terminus, for expression, and crystallized as a dodecamer; this construct is also catalytically inactive and motivated us to crystallize and solve the monomeric structure of the active, full-length soluble domain of *S. agalactiae* SrtA (PDB ID 7S56) [29]. We found that these C-terminal residues contain several hydrophobic residues which make intramolecular side chain interactions with amino acids elsewhere in the protein, likely explaining the lower stability and lack of activity of the truncated enzyme [29].

Structural implications for the sortase catalytic mechanism

Significant progress has been made in uncovering the molecular mechanism of SrtA-catalyzed transpeptidation; however, the precise details of this process remain unclear. To this end, the recent substrate-bound structures of spySrtA have offered some new insights, in particular regarding

Table 1. SrtA structures in the Protein Data Bank (March 2024)

Organism	PDB ID	Year released	Mutation?	Ligand?	Oligomeric state	Active state?	Method	Refs
Actinomyces oris	5UTT	2019	None	No	Monomer	Yes	X-ray crystallography	[35]
Bacillus anthracis	2KW8	2010	Contains N-terminal extension	No	Monomer	Yes	NMR	[36]
B. anthracis	2RUI	2015	Contains N-terminal extension	LPAT* peptidomimetic	Monomer	Yes	NMR	[26]
Lactobacillus rhamnosus	7CFJ	2021	None	No	Monomer	Unknown	X-ray crystallography	n/a
L. rhamnosus	7F87	2022	None	ALT tripeptide	Monomer	Unknown	X-ray crystallography	n/a
Listeria monocytogenes	5HU4	2017	None	No	Monomer	Yes	X-ray crystallography	[37]
Staphylococcus aureus	1IJA	2001	None	No	Monomer	No	NMR	[9]
S. aureus	1T2P	2004	None	No	Monomer	No	X-ray crystallography	[21]
S. aureus	1T2W	2004	None	LPETG peptide	Monomer	No	X-ray crystallography	[21]
S. aureus	1T2O	2004	Se-SrtA, C184A	No	Monomer	No	X-ray crystallography	[21]
S. aureus	2KID	2009	None	LPAT* peptidomimetic	Monomer	Yes	NMR	[25]
S. aureus	2MLM	2014	None	Benzo[d]isothaizol-3-one (inhibitor)	Monomer	No	NMR	[38]
S. aureus	6R1V	2019	None	2-(aminomethyl)-3-hydroxy-4-H-pyran-4-one prodrug	Monomer	No	NMR	[39]
Streptococcus agalactiae	3RCC	2011	Truncated	No	Dodecamer	No	X-ray crystallography	[41]
S. agalactiae	7S56	2022	None	No	Monomer	Yes	X-ray crystallography	[29]
S. agalactiae	7S54	2022	β7–β8 loop from *S. aureus* SrtA (with deltaN188)	No	Monomer	Yes	X-ray crystallography	[29]
Streptococcus mutans	4TQX	2015	Contains N-terminal helix	Tetraethylene glycol	Monomer	Yes	X-ray crystallography	[42]
Streptococcus pneumoniae	4O8L	2013	None	No	Domain-swapped dimer	No	X-ray crystallography	n/a
S. pneumoniae	4O8T	2013	C207A	No	Domain-swapped dimer	No	X-ray crystallography	n/a
S. pneumoniae	5DV0	2016	None	No	Domain-swapped dimer	No	X-ray crystallography	n/a
S. pyogenes	3FN5	2009	None	No	Monomer	Yes	X-ray crystallography	[43]
S. pyogenes	3FN7	2009	None	No	Monomer	Yes	X-ray crystallography	[43]
S. pyogenes	7V6K	2021	None	ML346 (inhibitor)	Monomer	Yes	X-ray crystallography	[44]
S. pyogenes	7S4O	2022	C208A	LPATS peptide	Monomer	Yes	X-ray crystallography	[28]

Table 1. (continued)

Organism	PDB ID	Year released	Mutation?	Ligand?	Oligomeric state	Active state?	Method	Refs
S. pyogenes	7S51	2022	C208A	LPATA peptide	Monomer	Yes	X-ray crystallography	[28]
S. pyogenes	7T8Y	2022	C208A	LPAT-lipid II, Thr-in	Monomer	Yes	X-ray crystallography	[28]
S. pyogenes	7T8Z	2022	C208A	LPAT-lipid II, Thr-out	Monomer	Yes	X-ray crystallography	[28]
S. pyogenes	8T8G	2024	C208A	LPALA	Monomer	Yes	X-ray crystallography	[33]
S. pyogenes	7S53	2022	β7–β8 loop from *L. monocytogenes* SrtA	No	Monomer	Yes	X-ray crystallography	[29]
S. pyogenes	7S57	2022	β7–β8 loop from *Enterococcus faecalis* SrtA	No	Monomer	Yes	X-ray crystallography	[29]
S. pyogenes	8SNQ	2024	F145E	No	Monomer	Yes	X-ray crystallography	n/a

the role of the conserved arginine residue (R216 in spySrtA) present in the Cys–His–Arg triad of sortase enzymes [28,33]. Prior work has suggested multiple roles for this arginine residue, including that it interacts with the backbone carbonyls of residues in the LPXTG motif (P4 Leu and/or P3 Pro), and possibly also makes specific contacts with the carbonyl and side chain hydroxyl group of the P1 Thr [25,26]. In this latter role, the interaction of the conserved arginine and P1 Thr has been suggested to constitute a substrate-stabilized oxyanion hole that stabilizes tetrahedral oxyanion intermediates encountered over the course of the transpeptidation reaction [26,27]. It should be noted that this function was first proposed based on structural work with *S. aureus* sortase B (saSrtB) bound to a substrate mimetic, and later suggested as a possibility for SrtA [1,26,27].

In the specific case of spySrtA, our recent substrate-bound structures suggest that the conserved Arg does not interact with the scissile P1 Thr carbonyl, but rather stabilizes the P4 Leu and P3 Pro backbone carbonyl atoms [28]. A similar orientation for the conserved arginine in saSrtA (R197) was also recently elucidated from NMR studies on the acyl-enzyme intermediate, providing additional evidence that this highly conserved residue primarily serves to position the P4 Leu and P3 Pro of the LPXTG motif [45]. Moreover, an engineered saSrtA variant with the ability to recognize the sequence LMVGG contains an R197S mutation, supporting the idea that this Arg is not directly involved in the catalytic mechanism and rather serves to hold the LPXTG substrate in the active site [46].

In place of the conserved Arg, an energy-minimized model of the acyl-enzyme intermediate in spySrtA suggests it is the side chain of T207, immediately preceding the catalytic C208 residue, and the backbone amide of H143, immediately following the catalytic H142 position, that interact with the P1 Thr carbonyl and likely stabilize oxyanion intermediates generated during the transpeptidation reaction [28]. This is supported by earlier molecular dynamics studies that found R197 (in saSrtA) does not generate an oxyanion hole to stabilize tetrahedral intermediates during the reaction [47], and is also consistent with the aforementioned recent NMR study that suggested a similar orientation for the carbonyl of the acyl enzyme intermediate [45]. A key role for the Thr (T207 in *S. pyogenes* SrtA and T183 in saSrtA) immediately preceding the catalytic cysteine is also supported by functional studies, in which mutations of these residues to alanine resulted in significant loss of enzyme activity [28,48]. It should also be

noted that Thr immediately preceding the catalytic cysteine is a highly conserved structural motif in the sortase superfamily [1,49].

These recent findings raise interesting questions about the currently defined catalytic triad of sortases, and support an updated proposal for the sortase mechanism (Figure 3). In this mechanism, which here is illustrated for the specific case of spySrtA, the P4 Leu and P3 Pro of the LPXTG motif are held within hydrophobic pockets in the substrate binding groove. These residues are further stabilized via hydrogen bonding with the conserved arginine (R216) of the Cys–His–Arg triad. The scissile P1 Thr is positioned for catalysis by interactions with the sidechain of T207, and the backbone amides of the catalytic cysteine (C208) and H143. As suggested for saSrtA, it is likely that the active form of spySrtA requires the catalytic cysteine (C208) and histidine (H142) to be in their thiolate and imidazolium form [50,51]. Nucleophilic attack from this active site thiolate generates a tetrahedral oxyanion intermediate that is stabilized by the side chain hydroxyl of T207 and backbone amide of C208 and H143. Collapse of this intermediate then generates the key acyl enzyme intermediate of the transpeptidation reaction (Figure 3, bottom right). From here, the incoming amine nucleophile of lipid II generates a second tetrahedral intermediate, which subsequently leads to the formation of the final transpeptidation product and release of the free spySrtA enzyme.

Structural studies of SrtA enzymes in complex with LPATX substrates or substrate mimetics have led to important mechanistic hypotheses concerning the details of the transpeptidation reaction. In some respects, similar features have been suggested, such as the positioning of the P4 Leu and P3 Pro residues in the active site of SrtA enzymes [25,28]. In other respects, mechanisms differ, such as in the precise role of the conserved arginine discussed in this review, and the extent to which this residue interacts with the P1 Thr or stabilizes transient oxyanion reaction intermediates. To that end, it should be noted that none of the structures reported to date have allowed for direct examination of these types of reaction intermediates, and mechanistic conclusions must therefore be drawn indirectly from structures and related models of complexes that mimic the acyl enzyme intermediate (PDB IDs 2RUI, and 2KID) or catalytically inactive mutants bound to peptide substrates (PDB IDs 7S4O, 7S51, 7T8Y, and 7T8Z). The number of SrtA structures including substrates or substrate mimetics also remains limited, and it cannot be ruled out that SrtA enzymes from different organisms may indeed exhibit differences in the details of their catalytic mechanism. Regardless, recent work involving spySrtA does suggest that additional clarification of the role of residues in the conserved sortase triad (Cys–His–Arg) is warranted, and further highlights the need for continued structural studies of enzymes from this superfamily.

Sortase enzymes in protein engineering

Alongside fundamental studies of sortase structure and function, work has also continued utilizing these enzymes as tools for protein engineering. Commonly referred to as SML, (Figure 1B), this chemoenzymatic strategy has seen widespread use over the ~20 years since it was initially reported [14]. While a comprehensive overview of SML applications is beyond the scope of this review, notable milestones in the development of this methodology include: site-selective labeling of protein C and N termini [14,52–55]; selective protein labeling on the surface of live cells or within living organisms [52,55–57]; the construction of polypeptides with unique topologies (circular, isopeptides, N-to-N, and C-to-C fusions) [53,58–62]; and the engineering of sortase variants with altered substrate specificities or enhanced catalytic activity [63–65]. Turning to recent uses of SML, a survey of the literature from 2021 to the present revealed over 100 examples of applications enabled by sortase ligations in the areas of therapeutics development, biomaterials, fundamental biochemistry, and cell biology studies. A nonexhaustive list of examples from this time period is given in Table 2, and select applications are discussed in more detail below. We also refer the reader to several excellent recent reviews focused on SML applications [16–20,66].

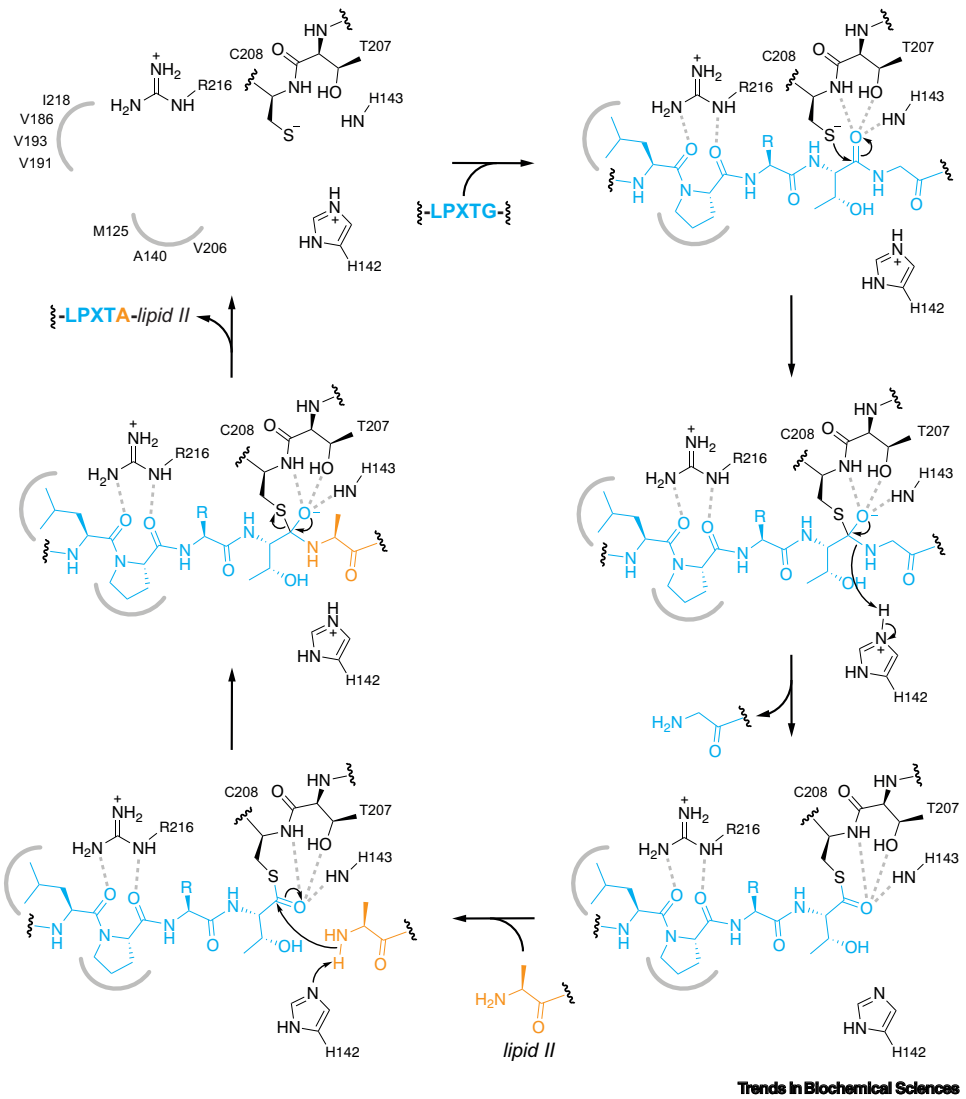

Figure 3. Detailed mechanism for sortase A (SrtA)-catalyzed transpeptidation. (top left) The SrtA active site (depicted for *Streptococcus pyogenes* SrtA) consists of hydrophobic pockets for the P4 Leu (I218, V186, V193, and V191) and P3 Pro (M125, A140, and V206). The side chains of highly conserved residues (R216, C208, and H142) are also critical for catalysis/binding, along with the backbone amides of C208 and H143. (top right) The binding of the LPXTG motif is facilitated by interactions between the conserved Arg (R216) and the backbone carbonyls of the P4 Leu and P3 Pro. The scissile amide bond of the P1 Thr residue is positioned for catalysis through interactions with the sidechain of T207, and backbone amides of C208 and H143. (middle right) Nucleophilic attack from the active site thiolate generates an oxyanion intermediate that is stabilized by the T207 sidechain, and backbone atoms of C208 and H143. Collapse of this intermediate, along with protonation of the departing Gly residue by H142, then generates the key acyl enzyme intermediate (bottom right). Subsequent nucleophilic attack (bottom left) by the N-terminal amino acid of the interpeptide bridge of lipid II (which is alanine in the case of S. pyogenes) leads to a second oxyanion intermediate (middle left). This intermediate collapses to form the final transpeptidation product (LPXTA–lipid II), along with release of free SrtA to continue the catalytic cycle.

As a specific example that underscores the therapeutic potential of SML, in 2021, Saunders *et al.* reported the development of a nanoparticle vaccine for coronaviruses that utilized SML to attach the ACE2-receptor binding domain (RBD) of the SARS-CoV-2 spike protein to the 24-subunit, self-assembling *Helicobacter pylori* ferritin nanoparticle [67]. In this case the saSrtA recognition sequence LPETGG was added to the C terminus of the SARS-CoV-2 RBD domain, and the

Table 2. Representative applications of SML (2021–March 2024)

Application	Enzyme	Refs
Selective modification of endogenous amyloid-β protein in human cerebrospinal fluid	SrtAβ[a]	[46]
Construction of ferritin nanoparticle vaccines for betacoronaviruses	SrtA5M[b]	[67]
Synthesis of polyubiquitin conjugates using SML and unnatural amino acid incorporation	SrtA5M[b], Srt2A-9[c]	[89]
Dual labeling of Fab fragments following CRISPR-mediated installation of orthogonal SML sites	Srt2a-9[c], Srt4S-9[d]	[73]
Attachment and regeneration of antithrombotic coatings for vascular grafts	SrtA5M[b]	[90]
Synthesis of β-adrenergic receptor with homogeneous posttranslational modifications	SrtA5M[b]	[91]
Modification of IgG lysine residues using proximity-driven isopeptide SML	Various[e]	[92]
Derivatization of nanobodies as imaging agents and therapeutics for influenza infection	SrtA7M[f]	[78]
Modification of the N-terminal tail of H3 histone *in vitro* and in live cells	SrtA6M[f]	[93]
Protein conjugation to spin-labeled probes for electron paramagnetic resonance spectroscopy	SrtA7+[f]	[94]
Attachment of nanobodies to GPCR agonists for modulating GPCR signaling	SrtA5M[b]	[95]
Crosslinking of collagen to form hydrogel matrices for tissues engineering	SrtA5M[b]	[96]
Conjugation of fentanyl to trypanosomes for eliciting an immune response	spySrtA	[97]
Controlled degradation of hydrogels for use in experimental cell culture platforms	SrtA5M[b], Srt2a-9[c], Srt4S-9[d]	[98]
Construction of C-to-C terminal fusion proteins	SrtA7M[f]	[62]

[a]Mutant variant of saSrtA with selectivity for noncanonical LMVGG motif.
[b]Catalytically enhanced variant of wild-type saSrtA containing five point mutations.
[c]Mutant variant of saSrtA with selectivity for LAXTG motif.
[d]Mutant variant of saSrtA with selectivity for LPXSG motif.
[e]A variety of fusions were evaluated consisting of wild-type or mutant variants of saSrtA fused to protein G or protein Z antibody binding proteins.
[f]Ca^{2+}-independent and catalytically enhanced variants of wild-type saSrtA with six (6M), seven (7M), or ten (7+) point mutations.

H. pylori ferritin particles contained a pentaglycine sequence at the N terminus of each subunit. The resulting conjugates (termed the RBD sortase-A-conjugated nanoparticle) successfully elicited an immune response in primates, even producing higher titers of neutralizing antibodies for certain SARS-CoV-2 variants as compared to lipid encapsulated mRNA vaccines [67]. A subsequent study found that these SML-derived nanoparticle vaccines could be expanded to display multiple viral antigens on the same particle, and as a result generate broadly protective immune responses in mice challenged with variants of betacoronavirus, sarbecovirus, and merbecovirus [68].

In addition to vaccine development, SML remains an attractive option for generating derivatives of antibodies and related proteins. One particularly powerful application is in the development of **antibody-drug conjugates (ADCs)**, wherein a therapeutic agent is targeted to specific tissues by covalently appending it to antibody or antibody fragments [69]. SML is well suited to the synthesis of these derivatives, which has commonly been achieved via site-specific transpeptidation at LPXTG motifs installed at the C terminus of antibody heavy or light chains [70]. Potent ADCs have been synthesized using SML, such anti-CD30 and anti-HER2 ADCs which recapitulated the activities of known cancer drugs, including brentuximab vedotin (Adcetris) and trastuzumab emtansine (Kadcyla), respectively [71]. In this case the SML-derived ADCs recapitulated the action of these drugs in non-Hodgkin's lymphoma and human breast cancer cell lines, as well as

a HER2-overexpressing ovarian cancer xenograft mouse model [71]. The SML strategy has also been extended to antibody fragments, with recent reports on the use of SML for derivatizing both Fab and Fc regions [72–74]. Along these lines, single domain nanobody proteins have frequently been modified using an SML approach. Recent examples include generating multivalent nanobody-quantum dot conjugates [75], the construction of targeted imaging agents [76–78], regulation of G-protein coupled receptor (GPCR) activation by conjugating nanobodies to peptide agonists [79], carbohydrate conjugation for cancer immunotherapy [80], and preparation of **nanobody–drug conjugates (NDCs)** loaded with neuraminidase inhibitors or corticosteroids to prevent severe influenza [78,81].

As a final recent highlight of SML technology, in 2021, Podracky *et al.* reported the successful evolution of a saSrtA variant with selectivity for a noncanonical substrate motif (LMVGG) that naturally occurs in amyloid-β (Aβ), an Alzheimer's disease-associated protein that is prone to aggregation [46]. This built on prior directed evolution studies from the same group that yielded saSrtA variants with enhanced catalytic activity (pentamutant saSrtA) or altered substrate preferences (LAXTG or LPXSG) [64,65]. Starting with a saSrtA variant (4S.6) that recognized LPESG, and using a similar yeast display platform in combination with fluorescence-activated cell sorting, a new variant (SrtAβ) was identified that exhibited a >1400-fold preference for LMVGG versus LPESG based on the activity of the enzyme displayed on yeast cells. [46]. This evolved enzyme (SrtAβ) contained an additional 25 mutations as compared to the 4S.6 starting sequence, notably including a mutation in the conserved Arg (R197S) as discussed above. In terms of utility, SrtAβ was used to label endogenous Aβ in samples of human cerebrospinal fluid, providing a sensitive assay for detection. In addition, SrtAβ was used to conjugate a hydrophilic peptide to a version of Aβ, $Aβ_{42}$, that forms fibrils, successfully impeding aggregation [46].

As a final comment on SML, it is intriguing to reflect on how future developments of this technology are likely to be informed by ongoing fundamental studies of sortase structure and mechanism. Examples of this are already present in the SML literature, such as in the case of an intramolecularly crosslinked saSrtA derivatives that exhibit heightened resistance to chemical and thermal deactivation [82,83]. In this case, the NMR structure of saSrtA (PDB ID IJA) was used to identify nearby sites for installation of mutant cysteine residues, which were subsequently used for crosslinker attachment. As an additional example, careful elucidation of the Ca^{2+} binding site in saSrtA informed the design of mutants (E105K and E108A/Q) that no longer required Ca^{2+} for activity [84]. These Ca^{2+}-independent mutations have been further combined with known rate-enhancing mutations of saSrtA to produce enzyme variants that are valuable for cell surface labeling, and for SML within *Escherichia coli* cells or *Caenorhabditis elegans* [57,85–87].

Looking ahead, we are also optimistic that a continued expansion in the scope of SML-compatible substrates beyond the standard LPXTG motif will be facilitated by additional structural work on the molecular basis for sortase substrate preferences. For example, the recognition that loops flanking the SrtA active site (β6–β7 and β7–β8) are critical for substrate scope has already guided directed evolution efforts and the production of chimeric SrtA variants that exhibit altered substrate preferences [22,32,63,88]. While the utility of many these alternate substrate sequences has yet to be established in the context of SML, this work does underscore the potential of SrtA enzymes as flexible platforms that could be further engineered to process a broader range of ligation partners.

Concluding remarks

The potential use of sortases in protein engineering applications is varied and extensive. The last 20 years has seen remarkable advances in tools created and biochemical assays utilized, including the development of novel protein derivatives with potential applications in vaccine development,

Outstanding questions

What is the exact role of the highly conserved Arg residue in sortases (R197 in *S. aureus* SrtA, R216 in *S. pyogenes* SrtA), and should this amino acid continue to be considered as part of the 'catalytic triad' of sortase enzymes?

What is the molecular basis for SrtA selectivity at the P1′ position of LPXTG motifs?

Beyond simply anchoring the enzyme in the bacterial membrane, does the transmembrane region of sortase enzymes play other roles in catalysis, for example by promoting colocalization with sortase substrates?

From the perspective of SML, what advantages do sortases from other subclasses (B–F) offer over SrtA variants, and can these enzymes be engineered to surpass the catalytic activity and versatility of SrtA enzymes?

Can sortase enzymes be engineered to eliminate ligation reversibility and limit the extent of competing hydrolysis?

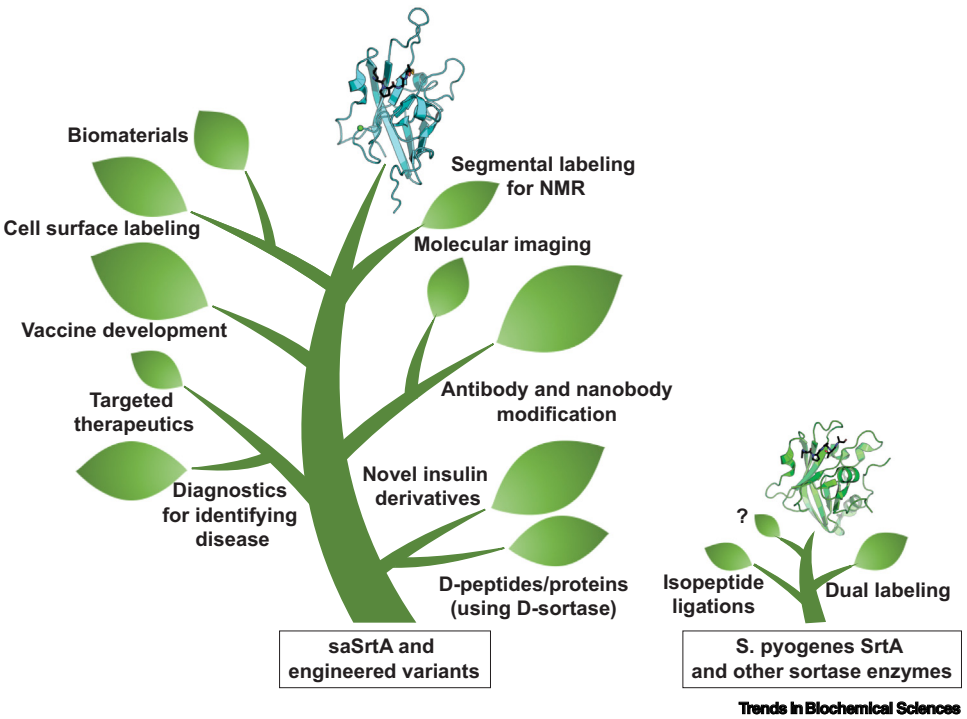

Figure 4. Advances and opportunities in sortase-mediated ligation (SML) strategies. This review discusses recent advances in SML applications, focused on class A sortases and specifically, on *Staphylococcus aureus* SrtA (saSrtA, PDB ID 2KID). As the tree on the left depicts, saSrtA and engineered variants have seen widespread use in biomedical and basic research applications [16–20,46,67,71,78,79,81,92,99–106]. As the tree on the right depicts, usage of *Streptococcus pyogenes* SrtA (PDB ID 7S51) and other sortase enzymes, including those from other classes, is less common [54,59,107,108], but represents a significant opportunity for growth, including continued progress and advances in SML technology.

cancer treatment, and neurodegenerative disease, as highlighted here (Figure 4). Critically, almost all SML applications to date utilized a single enzyme, saSrtA, and related engineered variants. Considering the varied specificities and activities of other class A enzymes, natural lack of calcium dependence (a unique feature of saSrtA), and differences in protein dynamics already observed, this entire field will benefit from future work to establish an expanded sortase toolbox for SML (see Outstanding questions) (Figure 4). Structural and functional studies of additional SrtA enzymes will undoubtedly aid in developing the next generation of sortase tools for protein engineering. We envision that a structural understanding of the molecular determinants of target recognition and catalysis can be used to design sortase enzymes suited for a range of target sequences. There are also several characteristics of sortases, including their membrane localization and sortases from the B–F families, which require further study. Although saSrtA was the first of this exciting enzyme family discovered, it should be but the edge of the sortase-mediated protein engineering frontier.

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Declaration of interests

The authors declare no conflicts of interest.

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