A Novel ψ-χ Fusion Protein for Unravelling the Contributions of χ to DNA Replication and Repair

AUTHORS

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ABSTRACT

Faithful DNA replication in *Escherichia coli* requires the DNA polymerase III holoenzyme (DNA pol III HE) and its clamp loader complex, which couples processive DNA synthesis with β_2 clamp loading. The clamp loader accessory subunits χ and ψ link single-stranded DNA-binding protein (SSB) to the replisome, stabilizing replication on SSB-coated templates and coordinating access for other SSB-interacting proteins (SIPs). Chi has also been implicated in tolerance to the chain-terminating nucleotide analog azidothymidine (AZT), though whether this function depends on χ within DNA pol III HE or on its independent interaction with the YoaA helicase remains unclear. To address this, we engineered ψ-χ fusion proteins with flexible glycine-serine linkers to tether the two subunits while preserving folding and activity. Both fusions were biochemically competent, supporting ATP hydrolysis and clamp loading on SSB-coated DNA. In vivo, however, neither the ψ-GS12-χ fusion nor expression of a ψχ operon restored AZT tolerance in Δ*holC* cells, whereas expression of χ alone was sufficient. Fusion expression also impaired growth in both WT and Δ*holC* backgrounds, a phenotype partially alleviated by disrupting χ-SSB binding. These findings support a model in which χ must dynamically engage SSB and YoaA outside of the clamp loader to promote AZT tolerance, highlighting the importance of regulated χ-SSB interactions in genome maintenance.

INTRODUCTION

The fidelity of DNA synthesis and repair reactions is critical for all cells to overcome replication errors. In *Escherichia coli*, most DNA synthesis is accomplished by DNA polymerase III holoenzyme (DNA pol III HE). This highly processive enzyme is an asymmetric dimer that coordinates replication of both the leading and lagging DNA strands at the replication fork.

The DNA pol III HE (reviewed in (1, 2)) consists of two distinct components: the pol III core polymerase and the clamp loader complex (Figure 1). The pol III core polymerases ($\alpha\varepsilon\theta$) actively synthesize the leading and lagging strands. The clamp loader complex ($\tau_2[\tau/\gamma]\delta\delta'\psi\chi$) coordinates the replisome by loading the processive β_2 sliding clamp onto DNA. Active clamp loaders contain three copies of the DnaX subunit, existing in two forms within the cell: the full-length form (τ), and the truncated form (γ), generated by a translational frameshift (3–5). The τ subunit contains an extended C-terminal domain that binds the DNA polymerase core and the DnaB helicase, physically coupling polymerase and helicase activities at the replication fork (3, 6). In contrast, γ lacks this domain and cannot tether DnaB, resulting in clamp loader with different structural and functional properties.

Active clamp loaders can be made using any combination of DnaX subunits (7). The γ complex clamp loader used in this study ($\gamma_3\delta\delta'\psi\chi$) lacks the C-terminal region of τ, which binds the core polymerase but is subject to proteolysis during purification in the absence of the polymerase. Multiple forms of the γ complex clamp loader can exist in vivo depending on the relative expression of τ and γ. The ability to assembly both types of clamp loaders enables dynamic regulation of replisome composition in response to cellular needs (3, 6, 8).

Within the clamp loader, the $\psi\chi$ complex functions as an accessory module to the DNA pol III HE and can exist as a heterodimer. Although χ is not essential for polymerase or clamp loader activity, it is the only known subunit of DNA pol III HE that makes direct physical and functional contact with the single-strand DNA-binding protein (SSB). Psi binds the clamp loader and χ, thereby linking SSB to DNA pol III HE and enhancing replication efficiency on SSB-coated DNA (Figure 1) (9–11). Chi is also important for efficiently loading clamps on DNA bound by SSB, emphasizing that SSB-clamp loader interactions hold instrinsic physiological significance even in the absence of the full DNA pol III HE (12).

In addition to SSB, χ has been shown to interact with YoaA, an XPD/Rad3-like helicase (Figure 1) (13, 14). The YoaA-χ complex is described as the likely physiological form of the helicase. Although *holC* (encoding χ) is not essential, *holC*-null mutants exhibit poor growth and rapidly acquire genetic suppressor mutations (15). Interestingly, deletion of *yoaA* in a *holC*-mutant background improves growth and viability, suggesting that YoaA is toxic in the absence of χ (16). Both YoaA and χ contribute to the maintenance of genome integrity, including roles in repairing methyl methanesulfonate (MMS)-induced lesions (17). Moreover, *yoaA* and *holC*, along with SSB-χ interactions, confer tolerance to 3′-azido-3′deoxythymidine (AZT) (Figure 1), a thymidine analog that blocks DNA synthesis by chain termination (16). Notably, the interaction between χ and ψ, which provides the only known connection to DNA pol III, is mutually exclusive with its interactions with YoaA (13). This raises the question of whether the role of χ in AZT tolerance depends on its association with DNA pol III, YoaA, or both (Figure 1).

The overarching goal of this study is to develop a tool that can be used to resolve the distinct contributions of χ to DNA replication and DNA damage tolerance. To do this, we engineered ψ-χ fusion proteins that can be uniquely incorporated into DNA pol III HE, leaving other pools of χ available for non-polymerase functions. We designed two fusions containing flexbile glycine-serine linkers, ψ-GS12-χ and ψ-GS8-χ, and biochemically characterized them as unique complexes and within the context of the γ complex clamp loader. The ψ-GS12-χ fusion was further tested in vivo to determine whether it could rescue the growth defect of a χ deletion strain and whether it contributes to AZT tolerance. This work establishes a stable and functional, novel ψ-χ fusion protein that operates within both the clamp loader and the DNA pol III HE. Importantly, the ψ-χ fusion provides a tool to distinguish between polymerase-associated χ from helicase-associated χ, enabling future studies to examine the dual roles of χ in DNA replication and repair.

MATERIAL AND METHODS

Protein Models

Structural models for Psi-GS12-Chi and Psi-GS8-Chi were made using AlphaFold Colab (https://colab.research.google.com/github/deepmind/alphafold/blob/main/notebooks/AlphaFold.ipynb) on January 20th, 2023, and January 31st, 2023, respectively. AlphaFold Colab runs a simplified version of AlphaFold v2.3.1 (18).

Strains and Media

The strains used in this study are listed in Table 1. *E. coli* strains were cultured in either lysogeny broth (LB) or Terrific Broth (TB) as specified. Culture media were amended with the appropriate concentration of antibiotics as recommended for each genetic vector (Table 1).

Cloning

The amino acid and nucleotide sequences for the GS8 and GS12 fusion are listed in Table 2.

The *holD*-GS12-*holC* was originally synthesized and cloned by GenScript into pUC57 between XbaI/BamHI. The terminal stop codon of *holD* was omitted, and the nucleotide sequence for GS12 was inserted immediately upstream of *holC*. pUC57_*holD*-GS12-*holC* was then transformed into *E. coli* DH5α and the plasmid extracted (Quiagen). To express *holD*-GS12-*holC* with *holB*, the plasmid was digested using XbaI (New England Biolabs, NEB) and BamHI (NEB). XbaI-*holD*-GS12-*holC*-BamHI was then ligated into MCS1 of pETDuet-1_*holB* using the sites XbaI/BamHI, creating pETDuet-1_*holD*-GS12-*holC*_*holB*.

GenScript also synthesized *holD*-GS8-*holC* and *holD*-GS12-*holC* in pET-15b using the restriction sites NcoI and BamHI to express the fusion proteins with a 6X N-terminal histidine tag. The terminal stop codon of *holD* was omitted, and the nucleotide sequence for GS8 or GS12 was inserted immediately upstream of *holC*.

To clone *holD*-GS8-*holC* into pETDuet-1, Gibson Assembly® (NEB) was designed according to the manufacturer's instructions. Fragments for *holD*-GS8-*holC* and *holB* were PCR amplified using Q5 High Fidelity Master Mix (NEB) following the manufacturer's instructions. *holD*-GS8-*holC* was amplified from pET15-b_*holD*-GS8-*holC* vector using the primers *holD*-GS8-*holC*_Fw and *holD*-GS8-*holC*_Rv listed in Table 2. *holB* was amplified from pETDuet-1_*holB* using the primers T7_LacO_RBS_*holB*_Fw and T7_LacO_RBS_*holB*_Rv listed in Table 2. pETDuet-1 was cut with AvrII (NEB) and NcoI (NEB) and gel purified (QIAquick® Gel

Extraction Kit, QIAGEN). The Gibson Assembly® Cloning Kit (NEB) was utilized according to the manufacturer's instructions to assemble fusion proteins into MCS1 of pETDuet-1 and *holB* into MCS2, creating pETDuet-1_*holD*-GS8-*holC*_*holB*.

Complementation vectors were generated using Genscript. The Shine Dalgarno (SD) sequence and spacer (AGGAGGaattc), based upon previous literature, were inserted upstream of each gene in the pBAD18 vector, and the complete constructs were cloned between the *KpnI* and *SphI* sites (19). The following inserts were cloned: *holD*, *holC*, a *holD*_*holC*_operon, with an SD sequence and spacer preceding each gene, and holD-GS12-holC. The plasmid pBAD18_*holD*-GS12-*holC* was used as the template to generate the R128A mutant by site-directed mutagenesis using the primers HolC_R128A_Fw and HolC_R128A_Rv listed in Table 2. Mutagenesis was performed using the Q5® Site-Directed Mutagenesis Kit (NEB) according to the manufacturer's instructions.

Protein Expression and Purification

The γ complex clamp loader ($\gamma_3\delta\delta'\psi\chi$ subunits) and β-clamp were expressed and purified as described previously (12, 20–22). Unlabeled protein concentrations were determined by measuring the absorbance at 280 nm under native conditions using extinction coefficients of 220,050 M^{-1} cm^{-1} for γ complex (23) and 14,700 M^{-1} cm^{-1} for β (21). All other protein concentrations were measured using a Bradford assay at an absorbance of 595 nm.

Expression of ψ-GS8-χ and ψ-GS12-χ. For ψ-GS8-χ, *E. coli* BL21 (DE3) cells were transformed with pET-15b_*holD*-GS8-*holC*. Cells were suspended in Terrific Broth supplemented with Carbenicillin 100 ng ml^{-1} (Carb100), grown at 37°C, shaking at 250 RPM until OD_{600} = 0.6. IPTG was added at a final concentration of 1 mM to induce protein expression, and cells were incubated at 37°C, shaking at 250 RPM, for 2 h. Cells were subsequently pelleted through centrifugation at 4°C, 6800 RCF for 30 min, decanted, and stored at -80°C. For ψ-GS12-χ, *E. coli* BL21 (DE3) cells were transformed with pET-15b_*holD*-GS12-*holC*. Cell growth and protein induction were done as previously described for ψ-GS8-χ.

Purification of ψ-GS8-χ and ψ-GS12-χ. Pellets of ψ-GS8-χ were resuspended in cell lysis/low imidazole buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 75 mM imidazole, 10% glycerol, 2 mM DTT, Pierce Protease Inhibitor Tablets – EDTA Free (ThermoScientific, Waltham, MA,

USA)) and lysed by two passes through a French Press cell disruptor. Lysate was clarified by centrifugation at 12,000 RCF for 45 min, and supernatant was applied to a HisTrap FF column (Cytiva, Marlborough, Massachusetts, USA). After washing with low imidazole buffer, bound protein was eluted using a linear imidazole gradient (75 mM to 500 mM). ψ-GS8-χ eluted in two distinct peaks, at approximately 210 and 310 mM imidazole. Elution fractions were pooled and dialyzed overnight against dialysis buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 20% glycerol, and 2 mM DTT), followed by a second dialysis in SP HP low salt buffer (50 mM Tris-HCl pH 7.5, 20 mM NaCl, 20% glycerol, and 2 mM DTT). The first elution peak retained from the nickel column was then applied to an SP HP column, where precipitation occurred during binding. Protein was recovered by elution with SP HP high salt buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 20% glycerol, and 2 mM DTT), re-purified through nickel affinity chromatography, and dialyzed sequentially in dialysis buffer and then overnight into storage buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 20% glycerol, and 2 mM DTT). The second peak from the nickel column did not go through the SP HP column and was dialyzed directly into storage buffer.

Pellets of ψ-GS12-χ were resuspended in cell lysis/low imidazole buffer and lysed as described above. Lysate was clarified at 12,000 RCF for 45 min, and supernatant loaded onto a HisTrap FF column (Cytiva). After washing with low imidazole buffer, protein was eluted using a linear imidazole gradient (40-500mM), eluting at approximately 210 mM imidazole. Fractions were dialyzed overnight in dialysis buffer and SP HP low salt buffer as described above. Protein was eluted using a linear salt gradient (20mM to 200mM), with ψ-GS12-χ eluting at two peaks, approximately 135 and 180 mM NaCl. Peak fractions were pooled separately, dialyzed overnight into storage buffer, and stored at -80°C as described above.

Expression of Clamp Loaders with ψ-GS8-χ and ψ-GS12-χ. For clamp loader containing ψ-GS8-χ, *E. coli* BL21 (DE3) cells were co-transformed with pETDuet-1_*holD*-GS8-*holC_holB* for the expression of ψ-GS8-χ and δ′, as well as pCOLADuet-1_*holA_dnaX* (22) for the expression of δ and γ. Cells were suspended in Terrific Broth supplemented with Carb100 and Kanamycin 50 ng ml^{-1} and grown at 37°C, shaking at 250 RPM until OD_{600} = 0.6. IPTG was added at a final concentration of 1 mM to induce protein expression and incubated at 37°C, shaking at 250 RPM, for 3 h. Cells were subsequently pelleted through centrifugation at 4°C, 6800 RCF for 30 min, decanted, and stored at -80°C. For the clamp loader containing ψ-GS12-χ, *E. coli*

BL21 (DE3) cells were co-transformed with pETDuet-1_*holD*-GS12-*holC_holB* for the expression of ψ-GS12-χ and δ′, as well as pCOLADuet-1_*holA_dnaX* for the expression of δ and γ. Cells were grown and induced as stated for clamp loader containing ψ-GS8-χ.

Purification of Clamp Loaders with ψ-GS8-χ and ψ-GS12-χ. Pellets of clamp loader ψ-GS8-χ were resuspended in cell lysis/low salt buffer (50mM Tris-HCl pH 7.5, 100mM NaCl, 5% glycerol, 2mM DTT, Pierce Protease Inhibitor Tablets – EDTA Free (ThermoScientific) and lysed as described above. Lysate was clarified at 12,000 RCF for 45 min, and the supernatant loaded onto an SP HP column (Cytiva). Because the clamp loader does not bind SP HP at 100mM NaCl, the flow-through was dialyzed overnight against Q HP low salt buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 10% glycerol, 2 mM DTT, 0.5 mM EDTA), then applied to a Q HP column (Cytiva). After washing with low salt buffer, the protein was eluted using a linear NaCl gradient (100 mM to 1 M) at approximately 460 mM NaCl. Collected fractions were dialyzed overnight against heparin low salt buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 10% glycerol, 2 mM DTT, and 0.5 mM EDTA) and applied to a HiTrap Heparin HP column (Cytiva). Bound protein was washed with low salt buffer and eluted with a linear NaCl gradient (50 mM to 1 M), with the protein eluting at approximately 245 mM NaCl. Pooled fractions were collected and dialyzed twice overnight against storage buffer (20mM Tris-HCl pH 7.5, 50 mM NaCl, 30% glycerol, 2 mM DTT, and 0.5 mM EDTA) and stored at -80°C.

Pellets of clamp loader containing ψ-GS12-χ were resuspended in cell lysis/low salt buffer identical to the ψ-GS8-χ clamp loader purification except that the sodium chloride concentration was lower (20 mM), and clarified as described above. The supernatant was applied to an SP HP column (Cytiva), washed with low salt buffer, and eluted with a linear NaCl gradient (20 to 300 mM) at approximately 140mM NaCl. Pooled fractions were dialyzed overnight against heparin low salt buffer and purified on a HiTrap Heparin column (Cytiva) as for ψ-GS8-χ clamp loader, eluting at approximately 230 mM NaCl. Fractions were dialyzed overnight against Q HP low salt buffer and applied to a Q HP column (Cytiva). After washing with low salt buffer, protein was eluted using a linear NaCl gradient (100 to 500 mM) at approximately 350 mM NaCl. Final pooled fractions were dialyzed twice overnight against storage buffer and stored at -80°C.

Differential Scanning Fluorimetry (DSF)

As the pH of Tris buffers varies more with thermal change than HEPES, WT ψχ and each fusion protein, ψ-GS8-χ and ψ-GS12-χ, were dialyzed twice against one liter of HEPES-based buffer (20 mM HEPES pH7.5, 50 mM NaCl) to remove Tris from the proteins. Dialyzed protein samples were centrifuged at 11,000 RCF for 5 min at 4°C to remove particulates, and protein concentration measured using a Bradford assay as previously described. The final protein concentration of 0.3 mg mL^{-1} was used for each reaction. Following the manufacturer's instructions, SYPRO Orange Protein Gel Stain (Invitrogen, Waltham, MA, USA) was diluted 1000X and the melting temperature (T_m) determined using a temperature range of 10°C to 95°C in increments of 0.5°C every 10 s. A real-time PCR device (CFX Real-Time PCR System, Bio-Rad) was used to monitor protein unfolding by the increase in the fluorescence of the fluorophore SYPRO Orange Assay. Melting temperatures were measured three times (n=3) with technical triplicates in each assay. Statistical significance was determined using a Student's T-test against the WT ψχ * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.

Circular Dichroism (CD)

WT ψχ and each elution peak from ψ-χ fusions were dialyzed against buffer containing 10mM KH_2PO_4 and 100mM KF to minimize buffer absorption in the far-UV spectra. Proteins were subsequently diluted to a concentration of 0.025 mg mL^{-1}. UV CD spectra (185-250 nm) were recorded at 25°C on a Circular Dichroism Spectrometer Model 400 (Biomedical, Inc., Lakewood, NJ, United States). A 1 mm path length cell was used at a step length of 1nm, an averaging time of 5s, a settling time of 0.333s, and a multi-scan wait of 1s. Spectra were corrected by subtracting the dialysis buffer measurements and normalizing by the mean residue weight (MRW) of the proteins, path length (P, cm), and protein concentration (C, mg mL^{-1}). Machine units (θ) were obtained in millidegrees from the spectrometer. The final spectra are expressed in Δε (M^{-1} cm^{-1}) using Equation 1.

Equation $$\Delta\varepsilon = \frac{\theta*(0.1*MRW)}{P*C*3{,}298} \quad (1)$$

ATPase-coupled Assay

ATPase activity was measured using a coupled enzyme assay measuring the decrease in NADH absorbance (24) as previously described (14) with slight modifications. Reactions were performed with 1μM 30-bp duplex with a dT_{30} 5' ss overhang (Table 2, YA1 and JH9T30). 80

nM of WT γ complex was run against 80 nM of γ complex containing ψ-GS12-χ or ψ-GS8-χ. The rate of ATP hydrolysis was calculated using Equation 2.

Equation $$rate = \frac{-\Delta(Abs_{340})/\Delta time}{(cuvette\ pathlength\ in\ cm)(6.22\frac{mM^{-1}}{cm})} \quad (2)$$

Labelling β with Fluorophores

Unique surface Cys residues were introduced into β (β-R103C/I305C/C260S/C333S) for clamp closing assays (25). Proteins were labelled as previously described (12, 25). Briefly, Cys residues were labeled with maleimide derivatives for Alexa Fluor 488 (AF488, Invitrogen) or Tetramethylrhodamine (TMR, Invitrogen), creating β-AF488$_2$ or β-TMR$_2$, respectively, and purified using a desalting column (BioGel P6-DG, BioRad) followed by anion exchange chromatography (HiTrap Q, Cytiva). Labelled β was further dialyzed to remove excess fluorophore. Labeled protein concentrations were determined by using a Bradford-type assay (Bio-Rad Protein Assay) with unlabeled proteins as standards.

Stopped-flow Clamp Closing Reactions

Reactions were performed according to Newcomb *et al*. with slight modifications. The decrease in AF488 fluorescence accompanying clamp closure on DNA was measured as a function of time using an Applied Photophysics SX20MV stopped-flow instrument. AF488 was excited at 495 nm using a 3.7 nm bandpass, and fluorescence emission was detected using a 515 nm long pass filter. The DNA substrate consisted of a 30-bp duplex and dT$_{30}$ 5′ ss overhang (Table 2, YA1 and JH9T30). A sequential mixing scheme was used in which clamp loader, β-AF488$_2$, and ATP were mixed and pre-incubated for 4 s prior to adding DNA and excess unlabeled clamp to measure rates of clamp closing. Final concentrations were 20nM of each clamp loader, 20 nM β-AF488$_2$, 200 nM unlabelled β, 80 nM DNA, and 1.5 molar equivalents of SSB per DNA molecule. For WT clamp loader and clamp loaders containing ψ-GS12-χ or ψ-GS8-χ, data were collected every millisecond over four seconds, for a total of 4000 time points. For the clamp loader Δχ, data was collected every millisecond over ten seconds, for a total of 10000 time points. Time courses were fit to double exponential decays (Equation 3) for WT and fusion clamp loaders to estimate the rates of decrease in fluorescence, where a_{fast} and a_{slow} are the amplitudes associated with the fast and slow phases, respectively, and k_{fast} and k_{slow} are the rates of the fast and slow phases, respectively,

and c is a constant. Double exponential decays did not improve the quality of empirical fits of time course reactions with $\Delta\chi$, so the simpler single exponential fits were used.

Equation $$y = a_{fast}\left(1 - e^{tk_{fast}}\right) + a_{slow}\left(1 - e^{tk_{slow}}\right) + c \qquad (3)$$

Fluorescence Measurements in Equilibrium Binding

Reactions were performed as previously described with slight modifications (25). AF488 was excited at 495 nm, emission spectra collected using a 3 nm bandpass, and relative intensity values calculated at 520 nm. Buffer background signals were subtracted from the spectra and relative intensities calculated by dividing intensities for solutions containing γ complex by the intensity for free β. The relative intensity of samples containing no γ complex was set to 1. K_d values were calculated using Equation 4, where β_o is the total concentration of γ_o is the total concentration of γ complex, and I_{max} and I_{min} are the maximum and minimum intensity, respectively.

Equation $$I_{obs} = \left(\frac{K_d+\beta_o+\gamma_o-\sqrt{(K_d+\beta_o+\gamma_o)^2-4\beta_o\gamma_o}}{2\beta_o}\right) \times (I_{max}-I_{min})+I_{min} \qquad (4)$$

Stopped-flow Clamp Opening Reactions

The increase in TMR fluorescence accompanying clamp closure on DNA was measured as a function of time using an Applied Photophysics SX20MV stopped-flow instrument. TMR was excited at 540 nm using a 5.6 nm bandpass, and fluorescence emission was detected using a 570 nm long pass filter. A single mixing scheme was used in which β-TMR_2 was mixed with variations of clamp loader. Final concentrations were 60nM of each clamp loader, 40 nM β-TMR_2, and 0.5 mM ATPγS. Data was collected every millisecond over ten seconds, for a total of 10000 time points. Time courses were fit to double exponential decays (Equation 3).

Complementation and Survival Assays

E. coli KEIO *holC* knockout strain JW4216 and the WT parent strain BW25113 were transformed with pBAD18 plasmids containing the empty vector, pBAD18_*holC*, pBAD18_*holD*, pBAD18_*holD*_*holC*_operon, pBAD18_*holD*-GS12-*holC*, pBAD18_*holD*-GS12-*holC*_R128A, and plated on LB + 0.2% glucose. Colony isolates were inoculated into liquid LB + 0.2% glucose and grown overnight at 37°C without shaking. Cultures were normalized to

OD_{600} = 0.05 in the same media and grown for 2 h at 37°C, with shaking at 250 rpm. Cells were then washed in LB, serially diluted, and spotted using 4 µL onto LB agar plates containing Carb100, either 0.2% glucose or 0.2% arabinose, and with or without AZT at 12.5 ng mL^{-1} or 25 ng mL^{-1}. Plates were incubated overnight at 37°C.

Buffers

Reaction buffers contained 20mM Tris-HCl pH 7.5, 50 mM NaCl, 8 mM $MgCl_2$, 0.5 mM ATP, 5 mM DTT, 0.5mM EDTA, 40 µg mL^{-1} BSA, and 4% glycerol.

Reproducibility and Error

Experiments were generally done three times. As an additional measure of robustness, measurements were made by comparing different reactions side-by-side (kinetic) to rule out systematic effects due to methods. Representative kinetic traces are shown. Rate and equilibrium constants were calculated from three independent experiments and are reported as the average with standard deviation. Titration data for each of the three replicate experiments are plotted along with the average and standard deviation.

RESULTS

Design of ψ-χ Fusions

Two ψ-χ fusion proteins were designed based on the solved heterodimer structures (PDB: IEM8 (26), 3SXU (27)). The distance between the C-terminus of ψ and the N-terminus of χ was measured using ChimeraX (~30Å). To account for missing residues within the structures (one in 1EM8 and four in 3SXU) and to facilitate accurate linker spacing within the globular protein structure, two linkers were designed: GS12 and GS8. Each consisted of glycine-serine repeats (Table 2), chosen for their flexibility and hydrophilicity, which promote solubility and reduce steric hindrance, as described in prior studies (28, 29).

Using AlphaFold Colab (18), models of ψ-GS8-χ and ψ-GS12-χ predicted that both GS8 and GS12 linkers were unstructured and of sufficient length to span the region between the C-terminus of ψ and N-terminus of χ. (Figure 2). Each ψ-χ fusion sequence was synthesized to ensure the rigor and viability of the fusion proteins *in vitro*.

ψ-GS12-χ is More Stable Than ψ-GS8-χ

To biochemically characterize the fusion proteins independently of the clamp loader, each fusion was expressed with an N-terminal 6X-His tag and purified using nickel affinity chromatography followed by cation exchange chromatography (Supplemental Figure 1). The ψ-GS12-χ fusion was successfully purified, yielding two distinct peaks on the cation exchange column. In contrast, ψ-GS8-χ eluted as two peaks from the nickel column, but was insufficiently soluble for further purification by cation exchange.

The thermal stability of the fusion proteins was assessed using differential scanning fluorimetry (DSF) using SYPRO Orange in HEPES buffer (Figure 3A-B). Both ψ-χ fusions exhibited higher melting temperatures than WT ψχ, consistent with stabilizing contributions from the linker (Figure 3A-B). For ψ-GS12-χ, both major peaks from purification displayed melting temperatures (T_m) ~5 ℃ higher than WT ψχ. For ψ-GS8-χ, the first and second purification fractions had a T_m of ~2.5 ℃ and ~1.5 ℃ higher, respectively. The ψ-GS8-χ fusion also exhibited greater variability in the T_m for the second peak, possibly indicating variations in protein folding or changes in the oligomerization state of the protein.

Circular dichroism (CD) was used to assess whether the fusions fold the same as WT. The CD spectra of each ψ-GS12-χ peak (Figure 3C) were nearly identical to one another and highly similar to that of WT ψχ, suggesting the fusion does not alter folding. However, ψ-GS8-χ precipitated under CD buffer conditions, preventing analysis. Collectively, these results demonstrate that the ψ-χ fusions are stable according to DSF, and the CD spectrum of ψ-GS12-χ closely resembles that of WT ψχ. However, the ψ-GS8-χ tended to be less soluble.

Ψ-χ Fusions are Biochemically Active in the Context of the γ Complex Clamp Loader

While the individual ψ-χ fusions were stable, evaluating their functionality within the clamp loader is crucial. To test this, ψ-χ fusions proteins were co-expressed with γ complex subunits to form γ complexes containing either ψ-GS12-χ ($\gamma_3\delta\delta'$ψ-GS12-χ) or ψ-GS8-χ ($\gamma_3\delta\delta'$ψ-GS8-χ) (Supplemental Figure 1).

Clamp loader activity was validated by comparing fusion-containing complexes to WT γ complex across several stages of the clamp loading process. The γ complex functions as a circular pentamer, where ATP binding by the γ-subunits drives conformation changes that

promote β clamp binding and subsequent DNA loading. ATP hydrolysis then triggers clamp release. ATP hydrolysis was measured using an enzyme-coupled assay that links ADP production to NADH oxidation (24). Each ψ-χ fusion γ complex exhibited an ATP hydrolysis rate similar to that of WT γ complex (25 μM min^{-1}), with γ complex containing ψ-GS8-χ or ψ-GS12-χ showing rates of 27 μM min^{-1} under the same experimental conditions (Supplemental Figure 2). Thus, incorporation of the ψ-χ fusions did not impair the basic ATPase activity of the clamp loader.

Because χ-SSB interactions facilitate clamp loading at ss/ds DNA junctions coated with SSB (12), fusion containing complexes were next assessed for clamp closing activity on DNA-SSB substrates (Figure 4A). The β sliding clamp was with Alexa Fluor 488 (AF488) at both sides of the dimer interface (12, 25). In this system, when β-$AF488_2$ closes on DNA, the AF488 fluorophores are within quenching distance of each other, resulting in low relative fluorescence (12, 25, 30). Conversely, when β-$AF488_2$ is open and bound to the γ complex, the fluorophores are separated, leading to high relative fluorescence. A DNA substrate consisting of a 30-bp duplex and dT_{30} 5′ ss overhang was used. The rates of clamp closing by the WT γ complex were compared to those of the γ complex without χ ($\gamma_3\delta\delta'\psi$) and with each fusion (ψ-GS12-χ or ψ-GS8-χ). Rates were measured using a sequential mixing stopped-flow, where the γ complex, sliding clamp, and ATP were mixed and pre-incubated (4s) to form a complex before the addition of DNA-SSB. An excess of unlabeled clamp was also added to limit reactions to a measurable single turnover event (12). While time courses for clamp closing are not exponential, data were fit to exponential decay to estimate the rate of decrease in fluorescence and measurements of reproducibility.

As expected, in the absence of χ (Δχ), γ complex was inhibited by SSB, with clamp loading occurring ~5-fold more slowly than WT (Figure 4B-C). In contrast, γ complexes containing either ψ-χ fusion closed clamps at rates indistinguishable from within experimental error (Figure 4C), demonstrating that both fusion proteins functionally interact with SSB to remodel DNA-SSB and clamp loading. The amplitudes of clamp closing reactions were reduced for the fusion complexes compared to WT, with γ complex containing GS8 showing the lowest amplitude.

To determine whether these amplitude differences arose from altered clamp-loader affinities, dissociation constants (K_d) for β-AF488$_2$ (20 nM) were measured in the presence of ATP (0.5 mM) (Figure 5A) (25). WT γ complex bound β-AF488$_2$ with a K_d of 2.0 ± 0.2 nM, consistent with previous values for β (3.2 nM, (31)) and β-AF488$_2$ (3.3 ± 0.2 nM, (25)). The ψ-GS12-χ γ complex exhibited a similar K_d of 2.1 ± 0.3 nM, while ψ-GS8-χ was 4.2 ± 2.7 nM (Figure 5B-C). At the concentrations used in stopped-flow clamp closing experiments, the differences in affinity change the fraction of clamp loader-clamp complexes from 73% for WT and ψ-GS12-χ clamp loader and 63% for ψ-GS8-χ, and as low as 56% when considering the standard deviation.

The maximal fluorescence intensities at saturation were equivalent, indicating that the same fraction of clamps was open in all cases. Thus, the reduced amplitudes observed for the ψ-χ fusions cannot be explained by differences in clamp opening defects and are at least partly attributable to the lower fraction of bound clamp loader-clamp complexes under experimental conditions, especially for ψ-GS8-χ. Amplitude differences also likely reflect subtle changes in clamp loader-clamp dynamics. To test whether clamp opening kinetics contributed to these differences, opening reactions were monitored during the pre-incubation step. Both ψ-χ fusion γ complexes opened clamps comparable to WT, and four seconds was sufficient for complete clamp opening (Supplemental Figure 3).

Neither WT ψ-χ or ψ-GS12-χ Rescues AZT Sensitivity of *E. coli* Lacking χ

The fusions demonstrated functionality *in vitro*, and the next objective was to assess their ability to function in vivo. Both fusion proteins function with the context of the clamp loader, but the ψ-GS8-χ fusion protein is generally less soluble, which precluded some measurements. Therefore, in vivo measurements were done with the ψ-GS12-χ fusion only. Strains lacking χ (Δ*holC*) are hypersensitive to AZT compared with WT strains, yet is unclear whether the role of χ in tolerance lies within pol III HE, the YoaA helicase, or both (16). To address this, Δ*holC* and WT *E. coli* strains were transformed with plasmids encoding *holD*-GS12-*holC* . Control plasmids enabled the expression of *holD* (ψ) alone, *holC* (χ) alone, or both *holD* and *holC* in tandem (*holD_holC*_operon), all under the arabinose-inducible pBAD promoter.

Expression of ψ-GS12-χ failed to rescue the AZT sensitivity of *ΔholC* cells, whereas expression of χ alone reduced AZT sensitivity to WT levels or better (Figure 6A). The *holD_holC*_operon, expressing WT ψ and χ, also failed to restore AZT tolerance, although it improved viability in the absence of AZT (Figure 6A and 6C). These results suggest that χ alone is sufficient for AZT resistance, and that ψχ complexes cannot substitute for this function. Importantly, this results implies that distinct χ-containing complexes may serve different roles, and that AZT tolerance specifically depends on χ outside of the clamp loader.

Dominant-Negative Effects of ψ-GS12-χ Fusion Depend on χ-SSB Binding

In doing the AZT tolerance experiments, we also observed that cells lacking *holC* and expressing ψ-GS12-χ exhibited poor growth compared to the empty vector control, characterized by reduced viable cell counts relative to WT (Figure 6D) and small colony morphology on plates (Figure 6E). The defect was partially alleviated by the expression of ψ-χ containing the χR128A mutation, which disrupts χ-SSB interaction, partially restoring viability (Figure 6C and 6E).

To determine whether these effects reflected general toxicity or stress-specific effects, the constructs were expressed in WT cells. Expression of ψ-GS12-χ impaired growth relative to the vector control (Figure 6F), although the effect was less severe than in the Δ*holC* background (Figure 6E). In this case, both the χR128A mutant and *holD_holC*_operon restored growth to WT levels (Figure 6F).

Fractional survival was reduced for ψ-GS12-χ, indicating a dominant-negative effect in the presence of endogenous χ and ψ, and a similar reduction was observed for the *holD_holC*_operon (Figure 6D). Under AZT stress (25 ng mL^{-1}), survival was reduced for all constructs, including the expression of χ alone. WT cells expressing ψ-GS12-χ showed the largest reduction in fractional survival, while the χR128A mutant and *holD_holC*_operon were closer to one another (Figure 6B). Expression of the ψ-GS12-χ fusion, χR128A mutant, and the *holD_holC*_operon all resulted in reduced total viability in WT cells (Figure 6D).

Together, these findings demonstrate that the ψ-GS12-χ fusion affects cell growth in both Δ*holC* and WT backgrounds, with the strongest effects observed under AZT stress. Disruption of the χ-SSB interaction partially alleviated these defects, underscoring the importance of

regulated χ-SSB binding for cell viability and DNA damage tolerance. While the fusion cannot substitute for χ in AZT tolerance, its distinct behaviour compared to χ alone suggests it may still serve as a tool to probe which χ-containing complexes are important in different cellular contexts.

DISCUSSION

The χ protein has a well-established role as a subunit of the DNA pol III HE, the main replicase in *E. coli*. Chi is an accessory subunit of the clamp loader portion of the holoenzyme and has no enzymatic activity on its own. Its primary role is to mediate physical interactions between the holoenzyme and SSB, which binds ssDNA that forms on the lagging strand during replication. Chi-SSB interactions stabilize the replicase on DNA, promote efficient DNA synthesis on SSB-bound DNA, and help remodel SSB on DNA to facilitate clamp loading (9–12). In additionally, χ has been implicated in DNA damage tolerance to AZT, a drug that stalls replication forks (16). Although χ is required for AZT tolerance, its precise mechanistic role remains unclear. Notably, AZT tolerance also requires the YoaA helicase, which interacts with χ in a complex that is independent of DNA pol III HE. This raises the possibility that χ contributes to tolerance either through its role in the clamp loader, its interactions with YoaA, or potentially through associations with other unidentified partner proteins. Consequently, it remains unclear which χ-containing complex is most crucial for function in vivo.

To address this question, we sought to distinguish the function of χ as subunit of the DNA pol III HE from its role within the YoaA complex in vivo. The binding sites for χ on DNA pol III HE and YoaA overlap, and amino acid substitutions in this region, such as χF64A, affect χ binding to both proteins (14, 16). This overlap complicates the use of point mutants to distinguish between the two χ pools. As an alternate strategy, we engineered a ψ-χ fusion that would be uniquely incorporated into the DNA pol III HE without affecting the pool of χ in the helicase. The optimal construct, ψ-GS12-χ, links the C-terminal end of ψ to the N-terminal end of χ with a 12-amino acid glycine-serine linker.

The design and function of amino acid linkers is a common strategy in protein engineering to preserve protein structure, function, and interactions (32). Gly-rich linkers, in particular, are naturally occurring separators that connect domains within proteins while allowing them to

retain discrete functions. These flexible linkers help stabilize interactions between protein partners, particularly when interactions are weak, transient, or involve disordered regions. Gly-Ser linkers are also relatively hydrophilic, which favors extended conformations over collapsed ones. By minimizing steric hindrance, they allow proper folding and function of the fused domains. In this study, our goal was to create a Gly-Ser linker between ψ and χ, providing sufficient flexibility to support stable domain interactions without compromising function both within the clamp loader or DNA pol III HE.

Both ψ-χ fusion proteins were expressed with an N-terminal His-tag and purified by chromatography. The His-tagged ψ-GS8-χ eluted from immobilized metal ion affinity chromatography (IMAC) in two separate peaks. SDS-PAGE analysis showed the two peaks contained the same polypeptide, suggesting that ψ-GS8-χ may adopt different conformations, folding states, or oligomeric states. Additionally, ψ-GS8-χ was poorly soluble and precipitated in several purification and buffer conditions, suggesting misfolding or aggregation. These observations indicate that the GS8 linker may be too short to maintain the correct intramolecular orientation between ψ and χ. As a result, the fusion may fail to form a stable heterodimer structure, as predicted by AlphaFold, and hinder the high-affinity binding of ψ to χ (Figure 7). When ψ is overexpressed alone, the protein is insoluble, suggesting that loss of ψ-χ contacts could further reduce solubility (33). Alternatively, oligomers may form if ψ from one fusion protein binds χ from another, generating higher-order assemblies that are less soluble (Figure 7).

In contrast, the ψ-GS12-χ fusion protein eluted as a single peak from the IMAC and was considerably more soluble than ψ-GS8-χ. Further purification by cation exchange yielded a doublet of closely spaced peaks, which may reflect conformational heterogeneity arising from the disordered region of ψ. CD spectroscopy confirmed that ψ-GS12-χ retained secondary structure similar to WT ψχ (Figure 3C), although CD cannot rule out multiple conformational states as illustrated in Figure 7. Importantly, DSF showed that both SP fractions of ψ-GS12-χ had identical T_m values, indicating that any conformational variability does not compromise stability. Moreover, the overall T_m of ψ-GS12-χ was significantly higher than that of WT ψχ, indicating a clear stabilizing effect of the GS12 linker (Figure 3A-B). This stabilization likely arises from improved spatial accommodation between the two domains, with the flexible linker relieving strain and allowing native-like intramolecular interactions

when compared to the GS8 linker. The fusion also ensures that ψ and χ are always held close in space, so when they transiently dissociate, they can rebind more rapidly through intramolecular association, which may further enhance stability compared to WT ψχ.

DSF analysis also showed that the Peak 1 of ψ-GS8-χ had tight error bars for T_m, indicating a reproducibly stable population, whereas Peak 2 displayed larger variability (Figure 3A). This suggests that a fraction of the ψ-GS8-χ population adopts a reproducibly stable conformation. However, the broader variability and reduced solubility of the GS8 fusion overall indicate that the shorter linker may not reliably support the correct folding or prevent aggregation.

Despite these structural differences, both fusion proteins retained functionality in the context of the clamp loader. ATP hydrolysis is a critical step in the clamp opening cycle, driving conformational changes required for opening and releasing the β clamp onto DNA. Clamp loaders that lack ψ are defective in some ATP-dependent steps, affecting both the promotion and the temporal order of clamp loading (34). Therefore, verifying ATP hydrolysis activity in the ψ-χ fusions is essential to establish that they functionally integrate into the clamp loader. In our assays, ATP hydrolysis rates for clamp loaders containing ψ-GS8-χ or ψ-GS12-χ were similar to those of WT (Supplemental Figure 2), indicating that the core ATP-driven conformational cycle is unaffected. Clamp loading assays further demonstrated that both fusions could remodel SSB-coated DNA and facilitate clamp closing (Figure 4A-C). This confirms that the fusions are incorporated into the clamp loader in a functional conformation, capable of engaging both SSB and DNA to mediate the structural transitions needed for clamp closure. However, although the rate of clamp closing was similar to WT, the fluorescence amplitude was reduced for both fusions, particularly ψ-GS8-χ (Figure 4A-C). The K_d values for clamp binding (Figure 5A-C) and opening kinetics (Supplemental Figure 3) were unchanged, suggesting that the reduced amplitude is not due to differences in clamp binding, which would otherwise reduce the fraction of clamp loader-clamp complexes. Moreover, equilibrium binding experiments showed that the increase in fluorescence observed for the fusion proteins was equivalent to WT, indicating that the same proportion of clamp loader-clamp complexes existed in the open conformation. These observations suggest that the reduced amplitude instead reflects subtle changes in conformational dynamics.

Overall, these findings confirm that the ψ-GS12-χ fusion is structurally stable, soluble, and functionally competent within the clamp loader. However, clamp loading and ATP hydrolysis assays only test core biochemical activities and do not reveal whether the fusion can support specialized roles at the replication fork, such as those required under DNA damage stress. To address this gap, we next examined whether ψ-GS12-χ could substitute for χ in vivo, focusing on its role in AZT tolerance. The expression of either the ψ-GS12-χ fusion or the WT ψχ operon did not rescue the AZT sensitivity of Δ*holC* cells (Figure 6A). In contrast, expression of χ alone restored AZT tolerance to WT levels or better, indicating that χ by itself is required AZT resistance. This requirement may reflect the need for the pool of χ that interacts with YoaA, or possibly with other unidentified binding partners, rather than clamp loader-associated χ. Importantly, the inability of ψ-GS12-χ to restore AZT tolerance does not mean that the fusion is nonfunctional in vivo, but instead suggests that different χ-containing complexes may serve distinct roles. The key question is therefore not only which χ-containing complex is most important, but also where χ exerts its essential functions in the cell.

In WT cells, expression of ψ-GS12-χ produced growth effects similar to those of the WT operon (Figure 6B and 6F), with both showing reduced fractional survival compared to vector controls. This suggests that these defects result from overexpression of ψχ complexes rather than from a fusion-specific effect. Introduction of the χR128A mutation, which disrupts the χ-SSB interface, partially alleviated growth defects in *ΔholC* cells (Figure 6A and 6E) and also reduced the small colony phenotypic defect of ψ-GS12-χ in WT cells (Figure 6F). These findings support the idea that χ-SSB binding is essential for maintaining normal cellular function.

Taken together, these findings indicate that while ψ-χ fusions are structurally stable and biochemically competent in vitro, their overexpression in vivo can be toxic. Toxicity may be linked to the stability and assembly properties of the fusion. Although ψ-GS12-χ was more stable than ψ-GS8-χ, the enforced tethering of the two subunits may still favor non-native assemblies. The fusions may also assemble into clamp loader subcomplexes that are not part of the full DNA pol III HE, consistent with evidence that clamp loader components can exist independently (9, 35, 36). These subassemblies, or free ψ-GS12-χ protein, could engage SSB or DNA in ways that compete with canonical replisomes.

Because only a limited number of DNA pol III complexes exist in each cell, not all ψ-GS12-χ fusions are expected to be incorporated into holoenzymes (37). A substantial fraction of the fusion protein is therefore likely free, where it may bind SSB inappropriately. Such improper binding could increase its local concentration at replication forks and interfere with normal protein handoffs. By tethering ψ to χ, the modularity of the clamp loader is reduced, which could trap χ on SSB and prevent access for other SSB-interacting proteins (SIPs) such as RecO, RecQ, PriA, or YoaA (38–41). In WT cells, χ normally engages SSB dynamically, allowing orderly exchange with downstream repair and restart factors. In contrast, the ψ-χ fusion could interfere with SIP recruitment, as supported by the dominant-negative phenotypes in vivo and the partial rescue by the χR128A mutation, which weakens χ-SSB binding.

Additional mechanisms may also contribute. Overexpression of both the ψ-GS12-χ fusion and the WT operon reduced viability in WT cells, suggesting that an increase of ψχ alone is harmful, regardless of the protein fusion (13). Elevated levels may alter clamp loader stoichiometry, titrate essential factors away from the replisome, or promote higher-order assemblies if the disordered region of ψ is engaging improperly. Persistent sequestration of clamp loader subunits or free ψ-GS12-χ on SSB-DNA would further reduce their availability elsewhere in the genome, slowing replication and increasing transcription-replication conflicts.

This model is further supported by live-cell imaging studies showing that SSB foci colocalize with DnaQ, DnaE, HolC, and HolD at replication forks (>90% colocalization), consistent with their established role as part of the gamma complex (42). These data reinforce that ψ and χ function directly at active replisomes where SSB is concentrated, providing the spatial context for our model. The dominant-negative effects of the ψ-GS12-χ fusion are therefore most likely to occur at replication forks, where persistent χ-SSB binding would block dynamic handoffs to other SIPs, including YoaA. Failure to recruit YoaA explains why neither the ψ-GS12-χ fusion nor the WT operon restored AZT tolerance in *ΔholC* cells, whereas expression of χ alone was sufficient.

The importance of this dynamic exchange is especially evident under AZT stress, which generates ssDNA gaps behind replication forks. Chi has been implicated in recruiting the YoaA helicase, which may facilitate gap processing and enhance AZT tolerance. Consistent

with this, deletion of *yoaA* improves growth and viability in *holC* mutants, indicating that YoaA activity is toxic when χ is absent (16). These genetic data suggest that χ may be essential for regulating or stabilizing YoaA, and that its absence leads to deleterious outcomes. The fusion may disrupt this regulatory role by anchoring χ to ψ and SSB, limiting its ability to engage YoaA when needed. Importantly, both the fusions and the WT operon are not restricted to clamp loader incorporation and may also exist as independent pools in the cell. In this context, persistent SSB binding by free WT or fusion complexes could further reduce opportunities for YoaA-χ interactions, providing a mechanistic explanation of why neither the ψ-GS12-χ fusion nor the WT operon restored AZT tolerance in Δ*holC* cells, whereas expression of χ alone was sufficient. In contrast, the R128A mutation reduces the χ-SSB occupancy, allowing YoaA or other SIPs to access SSB and partially restore tolerance.

Furthermore, the sequestration of clamp loaders on SSB-DNA may also diminish the availability of functional complexes elsewhere in the genome, slowing replication and increasing transcription-replication conflicts. These broad cellular effects are consistent with the impaired growth phenotypes observed even without added stress.

We propose a model in which modular interactions between χ, ψ, and SSB must remain flexible and tightly regulated to support replication. When this regulation is disrupted - whether by fusing χ to ψ, by altering expression levels, or by other perturbations - the modularity is lost, leading to inappropriate or persistent SSB engagement that disrupts normal clamp loader function and impairs DNA damage tolerance. Disruption of the χ-SSB binding interface alleviated these defects, highlighting the importance of regulated χ-SSB dynamics in preserving clamp loader integrity and replication fork stability.

DATA AVAILABILITY

Expression plasmids for proteins used in this study will be shared upon request to the corresponding author.

SUPPLEMENTARY DATA

AUTHOR CONTRIBUTIONS

K.A.P.P., S.T.L., and L.B.B. conceived and designed the project. K.A.P.P., J.D.G., M.J.P., and E.S.P.N. collected the data. K.A.P.P and L.B.B performed the analysis. K.A.P.P wrote the original draft. K.A.P.P, J.D.G., M.J.P., E.S.P.N., S.T.L., and L.B.B. reviewed and edited the draft. S.T.L. and L.B.B. supervised the project. L.B.B acquired funding for the project.

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CONFLICT OF INTEREST

None declared.

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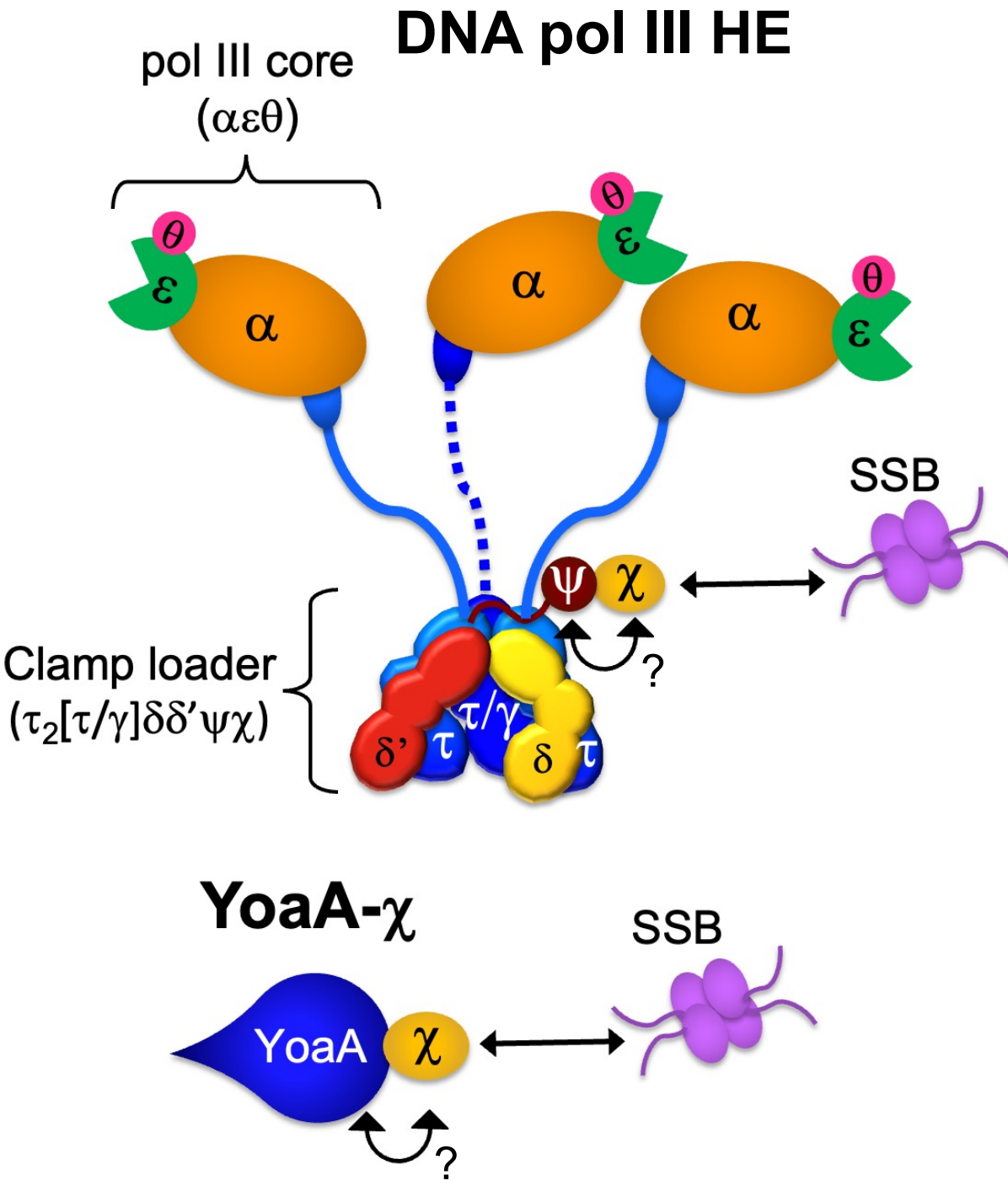
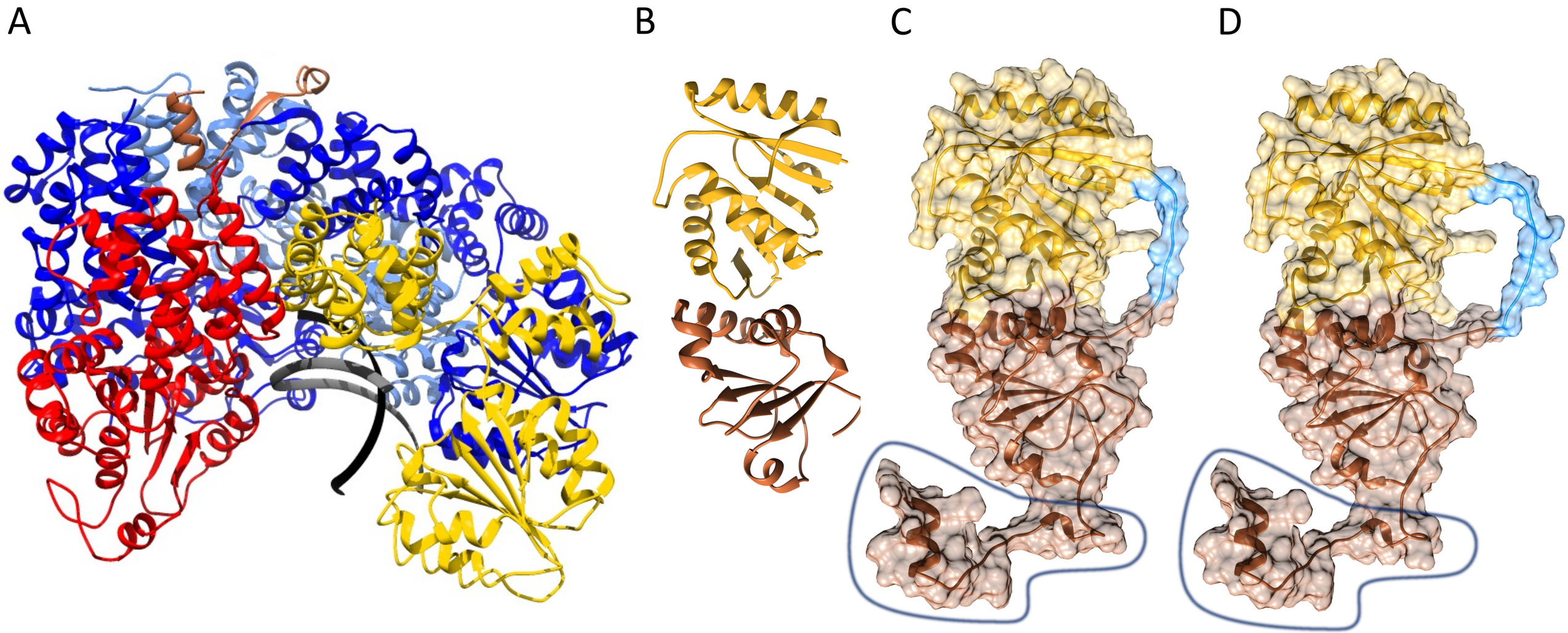
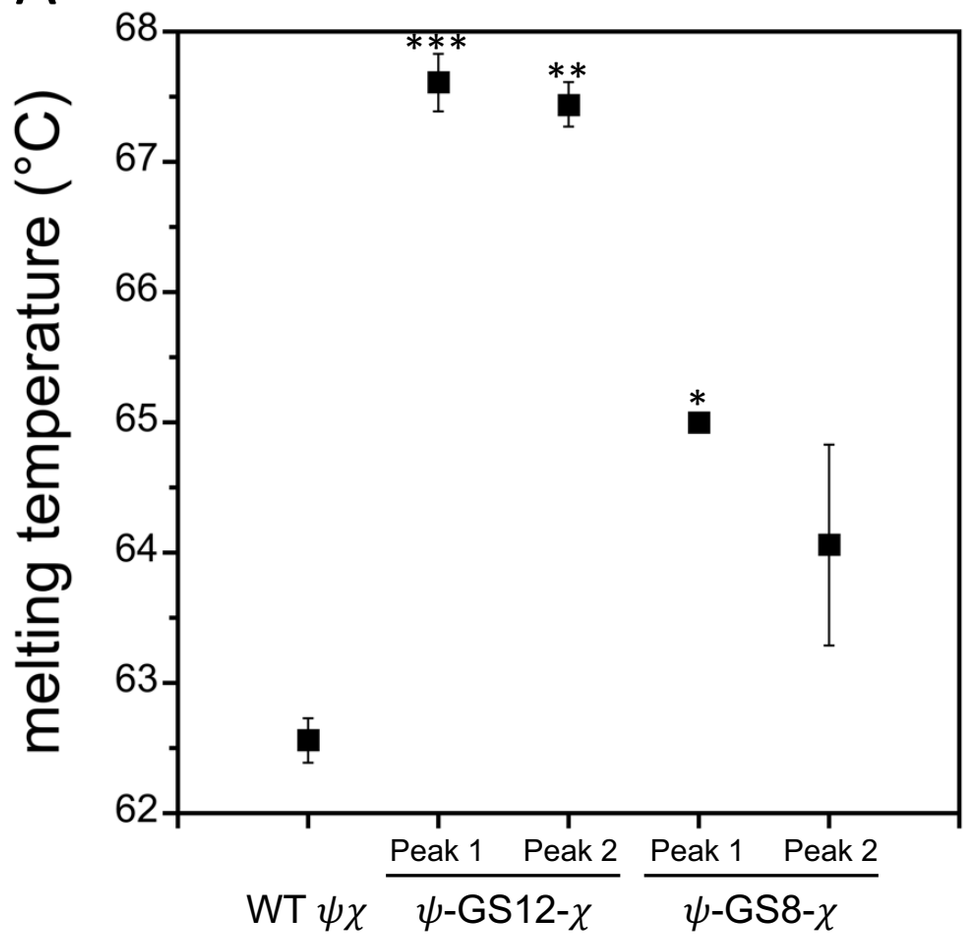


Schematic diagram of the DNA pol III HE (without β_2) and YoaA-χ. Interactions between χ and SSB are needed for AZT tolerance as indicated by arrows, but what part in each complex χ plays an important role is unknown.

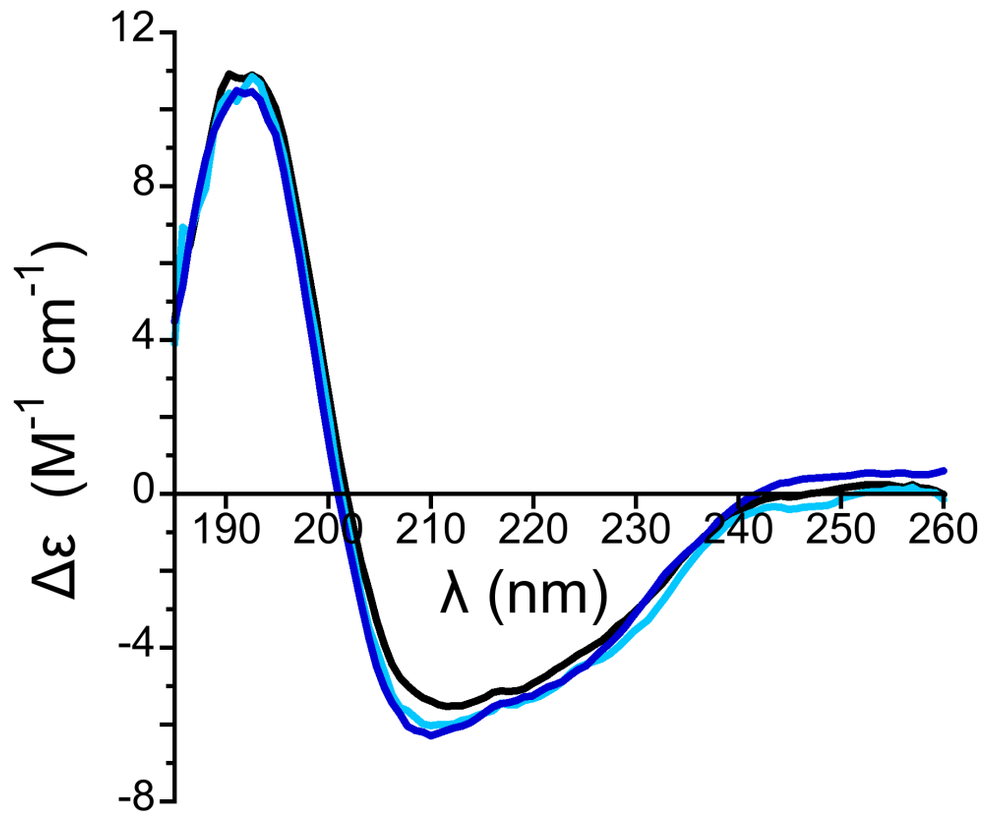


Ribbon diagrams of the WT clamp loader complex and ψ-χ fusion proteins. A) The clamp loader (PDB ID: 3GLI) ($\gamma_3\delta\delta'$, blue, yellow and red, respectively) is bound a peptide derived from the N-terminal of ψ (brown). The DNA primer and template strand are shown in grey. B) WT $\psi\chi$ (PDB ID: 3SXU) structure (ψ in brown and χ in gold), has 33 unmodeled residues in the N-terminal of ψ and 4 unmodeled residues in the N-terminal of χ. Fusion models made with AlphaFold 2 Colab for C) ψ-GS12-χ and D) ψ-GS8-χ have linkers shown in light blue. Circled region in C and D represent the peptide of ψ present in the clamp loader crystal structure in A.

A



C

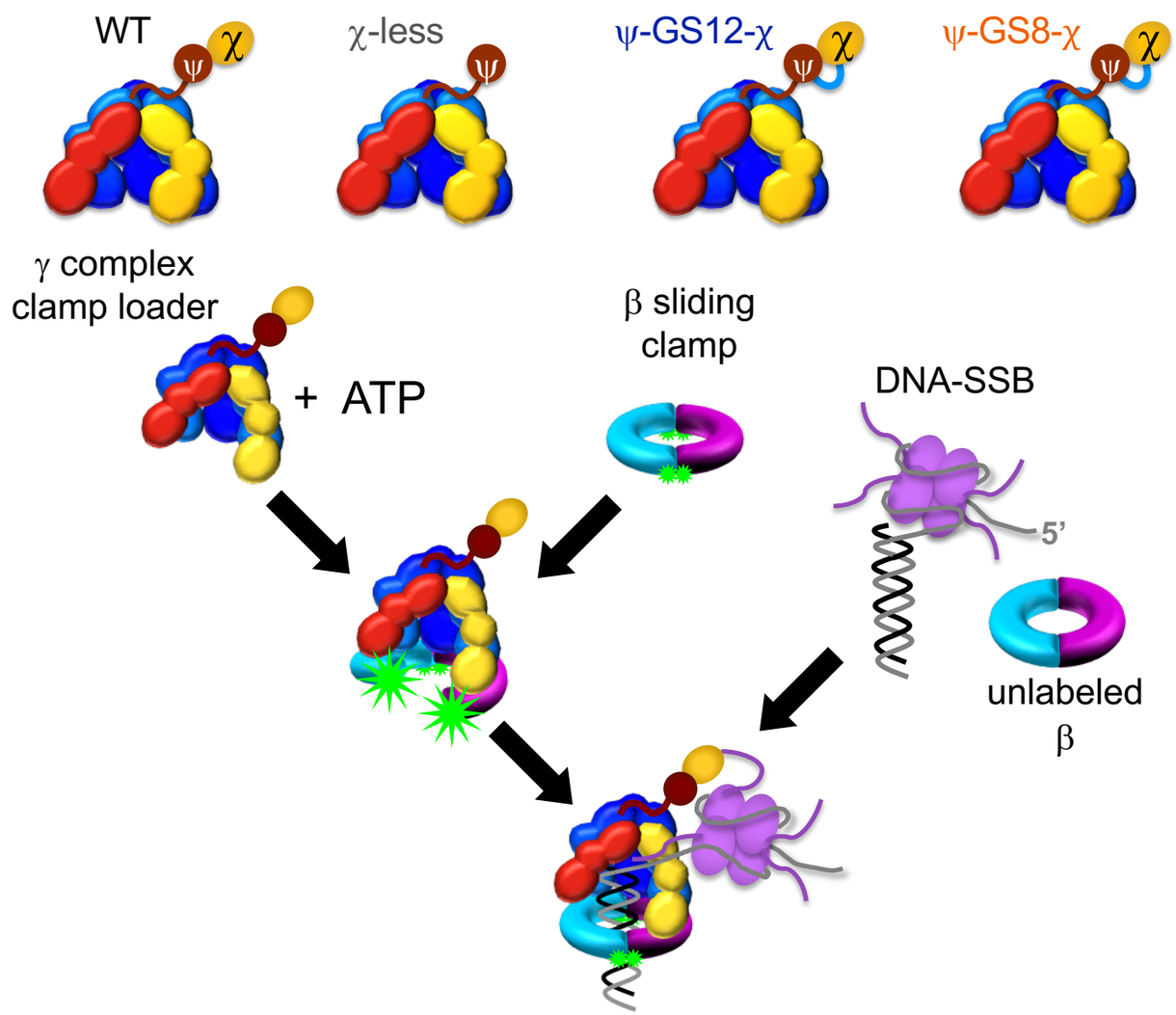


B

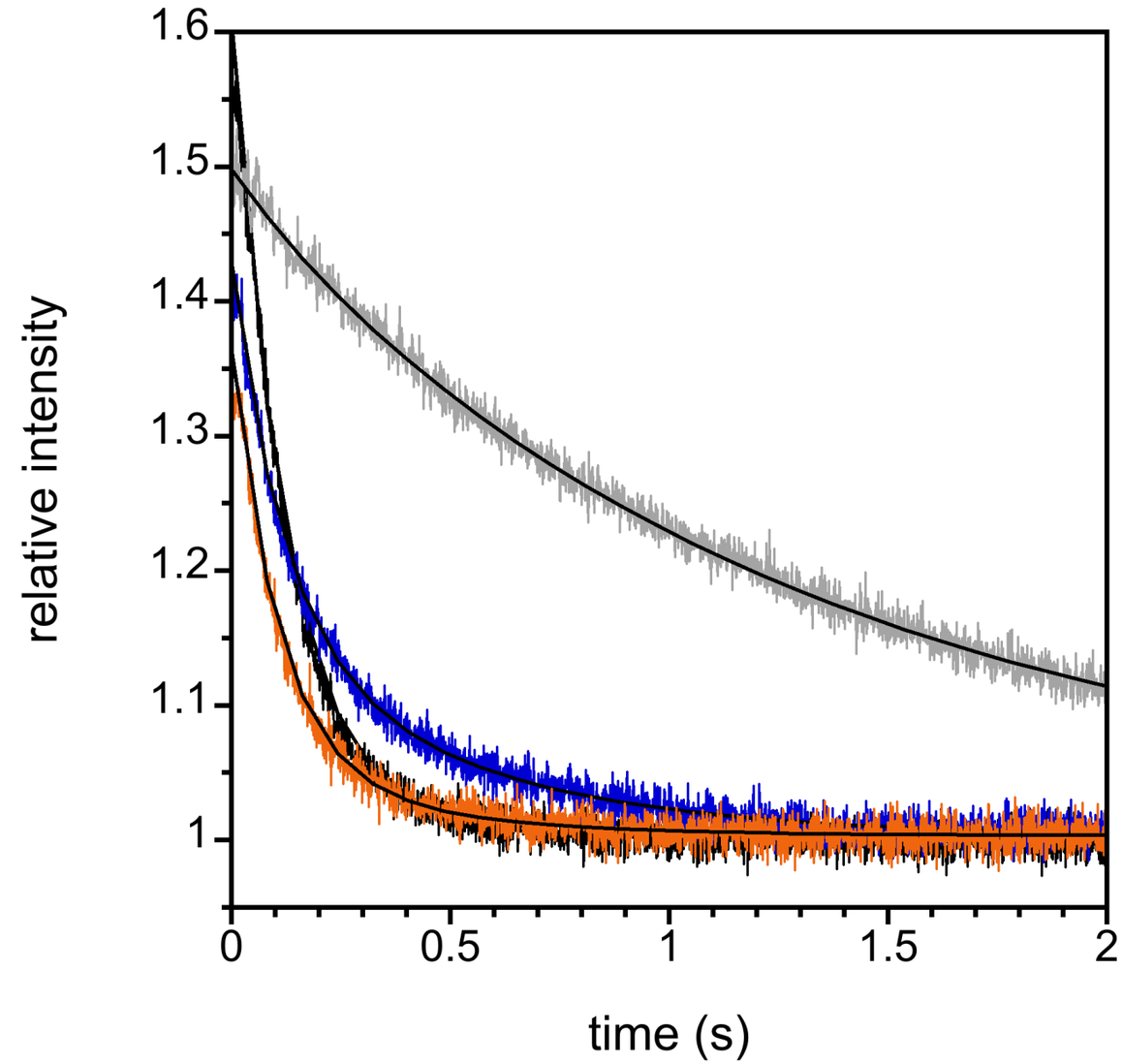
Protein	WT Ψχ	Ψ-GS12-χ		Ψ-GS8-χ	
Tm (°C)	62.6 ± 0.2	67.6 ± 0.2	67.4 ± 0.2	65.0 ± 0.0	64.1 ± 0.8

Modifications to $\psi\chi$ and their effects on protein stability. A) Melting temperature (T_m) of WT $\psi\chi$ and each fusion protein, ψ-GS12-χ and ψ-GS8-χ, was determined using SYPRO Orange and a temperature range of 10°C to 95°C in increments of 0.5°C every 10 s. Statistical analyses were performed using a students t-test against WT $\psi\chi$. *P < 0.05, **P < 0.005, ***P < 0.0005.B) Purified WT $\chi\psi$ (black) and ψ-GS12-χ peak one (dark blue) and two (light blue) analyzed by far-UV circular dichroism spectra. Each curve is an average of three scans, normalized by the mean residue weight of each proteins, path length, and protein concentration.

A



B

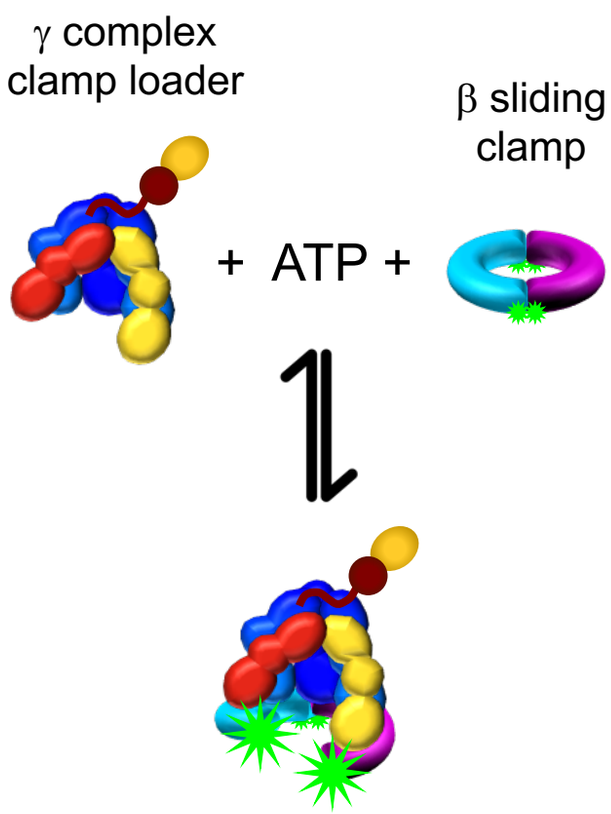


C

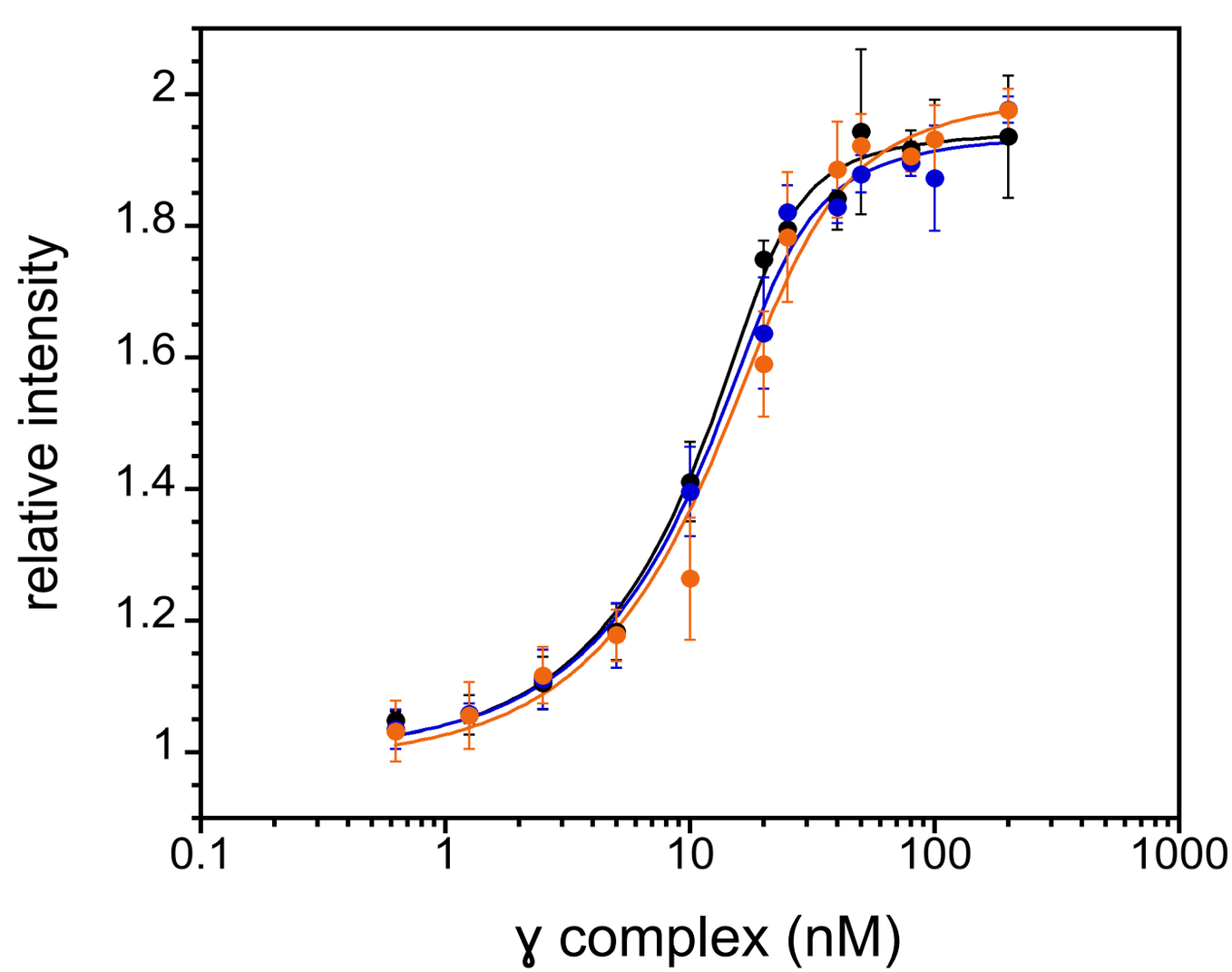
Clamp loader variation	a_{fast}	$k_{fast}(s^{-1})$	a_{fast}	$k_{fast}(s^{-1})$
WT	0.94 ± 0.03	9.26 ± 2.13	0.04 ± 0.02	0.90 ± 0.74
Δχ	0.69 ± 0.02	0.76 ± 0.09	-	-
Ψ-GS12-χ	0.49 ± 0.05	12.2 ± 5.89	0.21 ± 0.05	2.46 ± 0.51
Ψ-GS8-χ	0.36 ± 0.03	7.76 ± 1.25	0.08 ± 0.02	1.71 ± 0.70

Fusion clamp loading onto DNA. A) Clamp loading on DNA with bound SSB. The reaction scheme for clamp closing is shown alongside ɣ complex variations. The clamp loader was pre-incubated with β-AF488$_2$ and ATP for 4 s to form an open clamp loader-clamp complex prior to adding DNA-SSB along with an excess of unlabeled β-clamp to limit reactions to a single turnover. B) Representative clamp closing reactions for containing DNA-SSB along with 20 nM ɣ complex clamp loader, 20 nM β-AF88$_2$, 200 nM unlabeled β, and 0.5 mM ATP. ɣ complex variations are shown as WT (black), Δχ (grey), ψ-GS12-χ (blue), and ψ-GS8-χ (orange). Solid black lines through the data show fits to a double exponential (Equation 3) with values in Figure 4C.

A

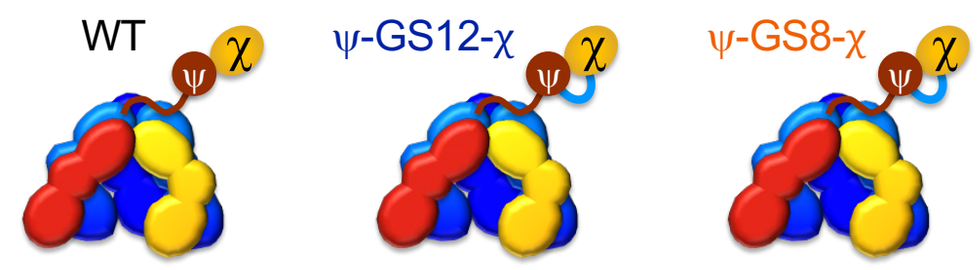


B

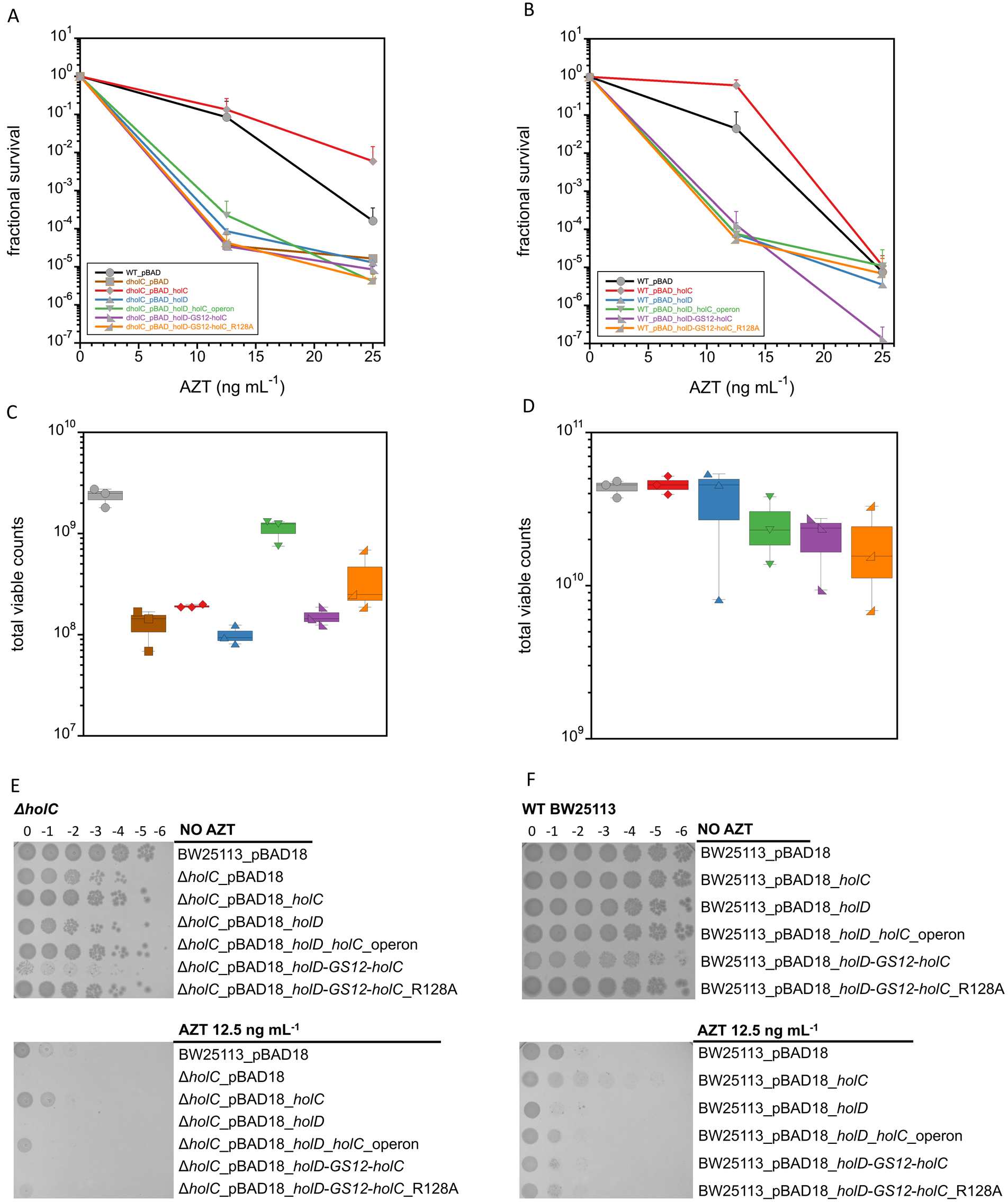


C

Clamp loader variation	K_d
WT	2.0 ± 0.2
Ψ-GS12-χ	2.1 ± 0.3
Ψ-GS8-χ	4.2 ± 2.7

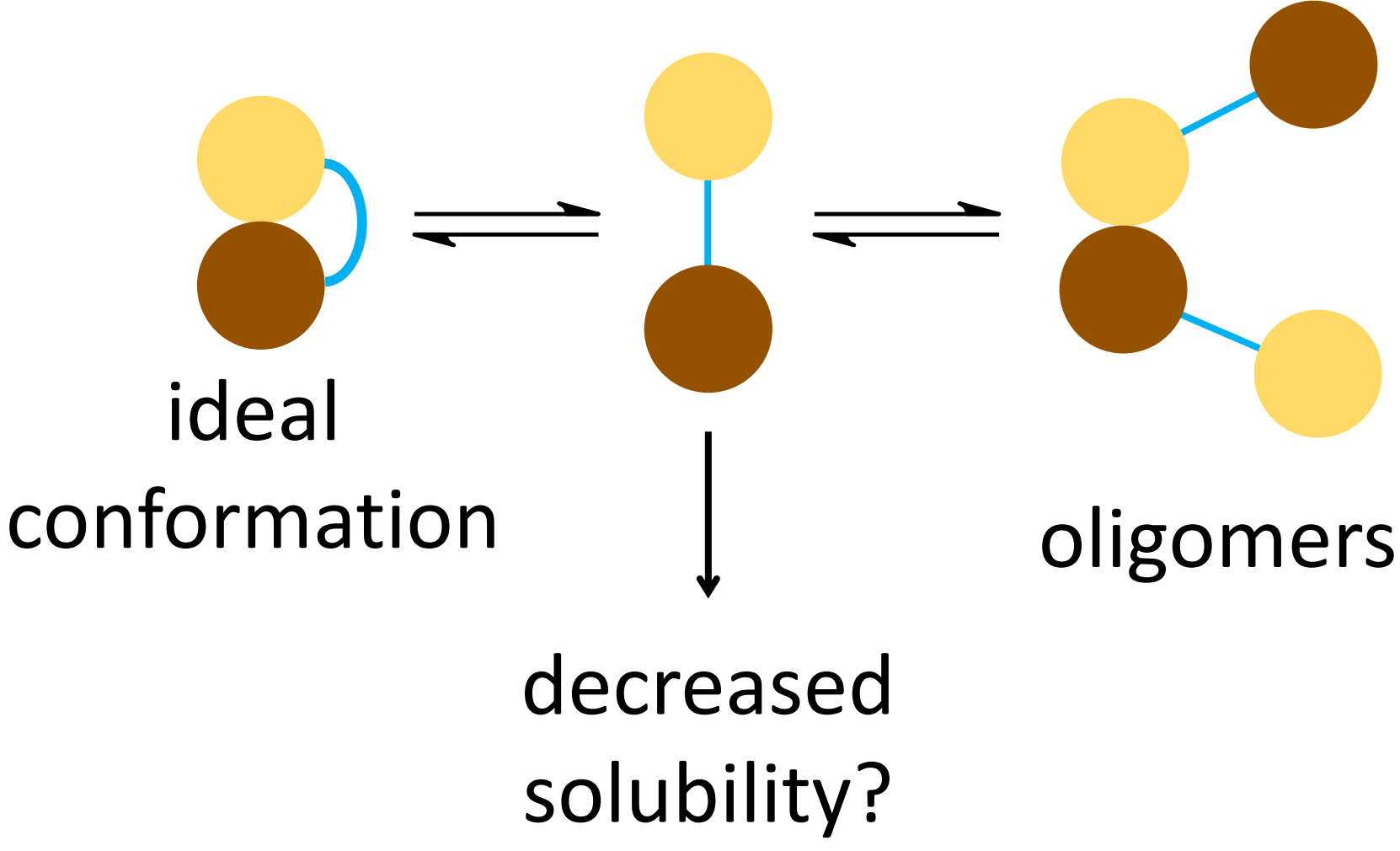


Equilibrium binding of clamp loader fusions to β. A) The reaction scheme for clamp opening is shown using WT γ complex the clamp loader is incubated with β-AF488$_2$ and ATP to form an open clamp loader-clamp complex. B) The relative intensity of AF488 at 520 nm is plotted as a function of γ complex concentration for solutions containing 10 nM β-AF488$_2$ and 0.5 mM ATP. γ complex variations are shown as WT (black), ψ-GS12-χ (blue), and ψ-GS8-χ (orange). C) Data from individual experiments were fit to Equation 4 to calculate the average dissociation constants.



Complementation of ψ-GS12-χ fusion into *E. coli* Δ*holC* and WT *E. coli*.

A) Fractional survival of *ΔholC* carrying the carrying the following plasmids: empty pBAD18 (square, brown line); pBAD18_*holC* (diamond, red line); pBAD18_*holD* (up triangle, blue line); pBAD18_*holD_holC*_operon (down triangle, green line); pBAD18_*holD*-GS12-*holC* (low left triangle, purple line); pBAD18_*holD*-GS12-*holC*_R128A (low right triangle, orange line), and WT *E. coli* carrying empty pBAD18 (circle, black line) as a control. B) Fractional survival of WT *E. coli* BW25113 carrying the same plasmids. Ten-fold serial dilutions of the indicated strains were plated on LB medium with or without AZT on 0.2% arabinose plates. C) Total viability of *ΔholC* carrying the carrying the following plasmids: empty pBAD18 (square, brown line); pBAD18_*holC* (diamond, red line); pBAD18_*holD* (up triangle, blue line); pBAD18_*holD_holC*_operon (down triangle, green line); pBAD18_*holD*-GS12-*holC* (low left triangle, purple line); pBAD18_*holD*-GS12-*holC*_R128A (low right triangle, orange line), and WT *E. coli* carrying empty pBAD18 (circle, black line) as a control. D) Total viability of WT *E. coli* BW25113 carrying the same plasmids. E-F) Ten-fold serial dilutions of the indicated strains were plated on LB medium with or without AZT on 0.2% arabinose plates. Data represent n=3 independent technical colony isolates plated in biological duplicates, shown as the average and standard deviation. Data represent n=3 independent technical colony isolates plated in biological duplicates, shown as the average and standard deviation. Representative images of plates are shown.



Model of fusion stability.
Schematic representation of proposed outcomes for ψ-χ fusions. In the ideal conformation, ψ and χ interact intramolecularly to form a stable complex resembling the native heterodimer. If the fusion prevents proper ψ-χ association, the protein may adopt a misfolded or partially unfolded state with decreased solubility. Alternatively, ψ and χ domains from different fusion molecules may interact intermolecularly, generating oligomeric assemblies that further reduce solubility.