

Glutamine Synthetase: Metals and Filament Coordination

Richard Sammy Muniz

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Reading Committee:
Justin Kollman, Chair
Michael Gelb
Kelly Lee

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Richard Sammy Muniz

University of Washington

Abstract

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Richard Sammy Muniz

Chair of the Supervisory Committee:

Justin Kollman

Biochemistry

Glutamine has a central role in cellular metabolism because it is a key donor of carbon and nitrogen atoms to other key metabolic precursors. Nitrogen fixation and mobilization is controlled through the inter-conversion of glutamate and glutamine by glutamine synthetase (GS). GS converts glutamate and ammonium into glutamine in an ATP-dependent reaction. GS assembles into filaments whose formation is conserved among *E. coli*, yeast, and plants. The higher-order structural organization observed among GS's and the importance of the glutamine pool within metabolism suggest these filaments may serve as a layer of regulation as seen with other filament-forming enzymes. Here we characterized in vitro assembly of *E. coli* and human glutamine synthetase. We show that the filament formation of *E. coli* GS is pH and divalent cation-dependent. Additionally, we show metals disrupt active site integrity through secondary structure changes. Finally, we formally show human GS can form filaments, define the residues involved in assembly, and provide structural evidence that these filaments bind and turnover substrates.

Acknowledgments

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Dedication

All of my accomplishments are dedicated to my siblings Anthony, Jaycob, Samantha, and George, my parents Angelina Muniz and Sam Muniz, my grandparents Manuel Mejia, Carlota Mejia, and Simona Muniz, and especially Pam.

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Chapter 1: Introduction

A: Glutamine and Glutamine Synthetase

Glutamine, measuring a concentration between 0.5-0.8 mM within blood plasma (Cruzat et al., 2018), is the most abundant amino acid in the human body and serves as a crucial inorganic nitrogen transporter and donor in all organisms (Castegna et al., 2018; Häberle et al. 2006, Unno et al. 2006). This is because glutamine is a precursor to numerous downstream processes in cellular metabolism such as nucleotide, amino acid, and amino sugar synthesis (Cruzat et al., 2018; Yoo et al., 2020, Unno, et al. 2006; Zhang et al; 2017). Glutamine synthetase is the only known protein to produce glutamine de-novo in mammalian cells (L. Cooper et al., 2012). It is an old, well-conserved enzyme essential for life (Kumada et al., 1993; de Carvalho Fernandes et al., 2022).

Glutamine synthetases are separated into three main subtypes, GSI, GSII, and GSIII, however, all share homo-oligomeric properties and have a conserved reaction mechanism (García-Domínguez, 1997) He et al. 2009; Joo et al., 2018; Krajewski et al., 2008; Moreira et al. 2019; Van Rooyen et al., 2011). The size and oligomeric state of GS can be used to distinguish the subtypes: on average GSIs are ~55 kDa protomers that form dodecameric assemblies, GSIIs are ~40 kDa protomers that form decameric assemblies, and GSIIIs are ~75 kDa protomers that, like GSIs, form dodecameric assemblies (Moreira et al., 2019; Müller et al. 2024). The decamer and dodecamer are composed of 2 stacked rings of 5 or 6 protomers, respectively. Additionally, the entire GS family shares two major domains, a beta-grasp domain near the N-terminus and a catalytic domain near the C-terminus (Eisenberg et al., 2000; Van Rooyen et al., 2011). Along the ring assembly, the active site is formed between protomers, the beta-grasp domain of one monomer interacts with the catalytic domain of the neighboring monomer. This creates 5 or 6 active sites per ring and 10 or 12 active sites per decamer and dodecamer, respectively (Eisenberg 2001, Krajewski et al., 2008, Moreira et al., 2019).

The molecular mechanism of glutamine formation by GS has been well characterized and is summarized in the review by Eisenberg et al. (2000) where they analyzed the structural and functional works of prokaryotic and eukaryotic GSs. The authors noted the following general GS characteristics: one, the active site between monomers is a bi-funnel that positions the substrates together at the pinch point and two, there are numerous divalent metal ions at the pinch point that serve to position the substrates for catalysis, assist in the reaction, and imparting stability to the greater oligomer. They also present the following reaction mechanism: first ATP enters from one end of the funnel, then glutamate and ammonium enter at the other end whereupon binding, ammonium is functionalized to ammonia, next ATP and glutamate react to form a gamma-glutamyl phosphate intermediate along with ADP, and finally the intermediate experiences nucleophilic attack by ammonia to produce glutamine and release phosphate.

The reaction funnel is in the same orientation along the pentameric and hexameric rings. Nucleotides enter and leave from one side, while amino acids and ammonia enter and exit from the other side. The amino acid and ammonium cycling side of rings come together to form the interface of the larger double ring structures in GSI and GSII, and are considered the bottom of the funnel (Eisenberg 2000, Krajewski et al., 2008) (Figure 1). Interestingly, GSIIIs have the opposite inter-ring orientation. Here the nucleotide-binding sides of the rings face each other with an interface coordinated by a GSIII-specific N-terminal extension (García-Domínguez et al., 1997; Moreira et al., 2019; Van Rooyen et al., 2011).

B: *E. coli* Glutamine Synthetase

E. coli GS is one of the most well-characterized GS's with biochemical and structural data spanning back nearly a century (Abell et al., 1991; Eisenberg et al., 2000; Joo et al., 2018, Meek and Villafranca, 1980; Shapiro & Stadtman, 1970; Stadtman, 2001; Valentine et al., 1968). *E. coli*, like most prokaryotes, expresses a GSI and forms a dodecameric catalytic unit. Additionally, *E. coli* GS has the characteristic GSI C-terminal helical thong that stabilizes the dodecameric structure by docking into a hydrophobic pocket of protomers on the adjacent ring (Eisenberg et al., 2000).

E. coli GS is heavily regulated with multiple levels of complexity. At the expression level, *E. coli* GS abundance in cells is directly altered by the availability of nitrogen in the organism's environment, and, at the protein level, *E. coli* GS experiences competitive inhibition, allosteric regulation, and post-translational, notably adenylation at tyrosine-398 (Shapiro et al., 1967; Stadtman, 2001, Stadtman, 2004). The adenylation at tyrosine-398 is a negative feedback response to high levels of glutamine, is reversible, and inhibits productive turnover of GS by sterically hindering glutamate's access to the active site at the bottom of the catalytic funnel (Shapiro et al., 1967; Okano et al., 2010). Compared to adenylation, the competitive inhibition and allosteric regulation of *E. coli* GS is very complicated. *E. coli* GS is downregulated by numerous downstream metabolites, including alanine, glycine, histidine, tryptophan, CTP, AMP, carbamyl phosphate, and glucosamine-6-phosphate, which bind competitively or non-competitively depending on the metabolite (Woolfolk, 1967). Interestingly, inhibition by any one inhibitor is incomplete, only in the presence of multiple inhibitors is *E. coli* GS completely inhibited (Woolfolk, 1967, Stadtman 2004).

Divalent cations also play an instrumental role in *E. coli* GS activity. *E. coli* GS requires divalent metal ions for dodecamer assembly and activity, with the specific metal ions within the active site affecting the specific activity and regulation of the enzyme (Abell et al. 1991, Denton et al., 1969; Hunt et al., 1975, Moreira et al., 2019). For instance, magnesium (Mg)-bound *E. coli* GS is inhibited by adenylation, while manganese (Mn)-bound *E. coli* GS maintains activity (Moreira et al., 2019). However, after thorough enzymatic analysis by Abell et al. (1991), the authors state the Mg-bound form is the most optimized catalytically and therefore most likely the

biologically relevant form. Still, the complex regulation of *E. coli* glutamine synthetase has made it a target of investigation and a pristine example of enzyme tuning.

C: Human Glutamine Synthetase

Humans express a GSII, which is predominantly found in eukaryotes (Krajewski et al., 2008; Moreira et al., 2019; Petrovska et al. 2014), and while some eukaryotes, like plants, express at least two tissue-specific isoforms of GSII (Unno et al. 2006), mammals only express one. The lone human GSII is ubiquitously expressed in all cell types and any mutations that impact activity lead to death shortly after birth. (Häberle et al. 2006; Vermeulen et. al., 2008) This vulnerability highlights the importance of GS for human homeostasis. For example, in the brain, it is tightly linked to the GABA pathway and highly expressed in astrocyte cells for glutamate-glutamine cycling. In other organs and cells, human GS assists with toxic ammonia removal (Suárez et al. 2002), and pH regulation (Frieg et al, 2021).

Recently, human GS has been identified as a cancer therapy due to the cancer microenvironment's heavy dependence on glutamine for increased amino acid and nucleotide production (Kim, 2021, Zhao et al., 2022). This has prioritized a deeper understanding of human GS function and regulation for drug exploitation (Kim et al, 2021; Jin et al., 2023). The human GS enzyme does not experience regulation through adenylation as the GSI proteins, however it does contain an N-terminal degron region. This initial 24 amino acid region activates the ubiquitin-proteasome system upon acetylation of Lys11 and Lys14 leading to the degradation of human GS (Chek et al, 2021). Chek and colleagues also showed that the product of GS, glutamine, increases the acetylation of Lys11 and Lys14, supporting a model for feedback inhibition.

A peculiar trait of human GS is the enzyme's moonlighting functions. In one case, the five surface and solvent-exposed K52 residues on one side of the human GS decamer interact with BEST2, an ion channel, in a glutamate-dependent manner to regulate the ion channel's transport (Owji et al, 2022). Additionally, human GS was shown to palmitoylate RhoJ and alter downstream angiogenesis (Eelen et al. 2018). Furthermore, Zhao et al. (2022) recently found that human GS interacts with the nuclear pore protein NU-88 and promotes tumor cell proliferation. The role of GS in these interactions is independent of its bio-synthetic function (Eelen et al. 2018; Zhao et al. 2022). This illustrates that although human GS has been fairly well-characterized, there is still much to learn about its function in the greater cellular context.

D: Filaments in Metabolism

Metabolism is an immense network of interconnected biochemical pathways with many subdomains governed and dependent on the interaction of proteins with one another. Over the years, as tools for the direct visualization of these interactions advanced, it became clear that macromolecular assemblies play a key role in the activity of numerous metabolic pathways (Park

and Horton, 2019). Importantly, the ability of a lone enzyme to homo-oligomerize is conserved across all domains of life (Elad, 2017). Additionally, it has been shown that proteins evolved with the capacity to readily form macromolecular assemblies with as little as a single amino acid substitution (Elad, 2017). The conditions that bring about filament formation vary across proteins and species. Studies with enzymatic analysis have shown filaments impose an additional level of regulation on enzymes (Park and Horton 2019; Burrell et al., 2022; Calise et al., 2024; Hansen et al., 2021; Hvorecny and Kollman, 2023; Hvorecny et al., 2023; Lynch et al., 2017; Lynch et al., 2020). Filaments have been shown to affect specific activity, cooperativity, substrate binding, and the binding of allosteric regulators (Simonet et al., 2020). Importantly, filament assembly and enzyme regulation in glutamine-glutamate cycling has recently been identified (Adamoski et al., 2023; Amanda Tragueta Ferreira et al., 2013; Cassago et al., 2012; Feng et al., 2024; Guo et al., 2024; Jiang et al., 2022). Adamoski et al. (2023) and Ferreira et al. (2013) showed that glutaminase in humans, the enzyme that converts glutamine back to glutamate, forms filaments in the presence of the known glutaminase activator phosphate. Additionally, the authors show that filament formation reduces k_m for its substrate, glutamine, and enhances mitophagy protection. Glutaminase filaments regulate one side of glutamate-glutamine cycling. Evidence showing GS forms filaments *in vitro* and *in vivo* supports that filaments impart an extra layer of regulation on this entire system.

E: Evidence of Glutamine Synthetase Filaments

Despite their differences, GS subtypes share numerous characteristics, most interestingly the capacity of GSI and GSII to form larger superstructures. Some of the first electron micrographs of GS from the mid-20th century showed *E. coli* GS formed filaments and larger bundles in the presence of divalent cations (Valentine et al., 1968; Frey et al., 1975). However, this higher-order assembly was primarily ignored, as tools to investigate these structures with high resolution were limited (Park and Horton, 2019; Carroni et al., 2016). Since their initial discovery, GS filaments or evidence of larger GS assemblies have been observed in other prokaryotes, yeast, archaea, and humans (Chek et al, 2021, Data, P., 2022; He et al., 2009; Herdering et al., 2024; Huang et al., 2022; Petrovska et al, 2014). These discoveries suggest that GS filament assembly is widespread across the domains of life.

In *E. coli* GS, specific divalent metal supplementation, growth media conditions, and purification conditions were all shown to influence GS filament assembly (Frey et al., 1975; Shapiro et al., 1967; Valentine et al., 1968). Huang et al. (2022) produced the first cryo-EM structure of *E. coli* GS filaments under nickel (Ni) and found histidine residues at the N-terminus were involved in the assembly. In yeast, stress conditions including starvation and acidic pH stimulated robust GS assembly *in vivo* leading to inactivity (He et al., 2009; Petrovska et al, 2014). Finally, higher-order assemblies of human GS have been seen in cryo-EM micrographs with BEST2 (Owji et al, 2022), protein samples purified from brain microsomes (Tringides et al., 2022), and under conditions that mimic acetylation (Chek et al. 2021).

Fascinatingly, the exact conditions that bring about GS filament assembly vary across and among species. Only *E. coli* GS is known to directly require divalent cations for filament assembly, while yeast GS filaments respond to metabolic demand and environmental conditions (He et al., 2009; Petrovska et al, 2014). Human GS filaments have only been serendipitously and sporadically observed, so the conditions that stimulate filament formation and their structural impact are unknown. Additionally, filament formation has not yet been seen in GSIII proteins, although as a recently identified subtype, there is limited information and few examples of GSIIIs currently available (García-Domínguez et al., 1997; Van Rooyen et al., 2011).

Evidence of GS filaments in multiple species with varied requirements for assembly in GSIs and GSIIIs suggests filaments are a conserved trait. Additionally, the architecture of filament formation and the structural effect on the catalytic GS unit have not been fully investigated in *E. coli* or human GS. Here, we analyzed the filament structures of human and *E. coli* GS. Using electron microscopy as our primary mode of investigation we determined the conditions and extent of filament assembly in *E. coli* GS under various divalent cation conditions. Additionally, for both human and *E. coli* GS, we tested the impact of substrates and pH on filament assembly. To probe the structural impact of filament assembly, we used cryo-electron microscopy to determine high resolution structures of *E. coli* and human GS with and without ligands. With cryo-EM, we identified the residues that bind Ni, Co, and Zn cations in the *E. coli* GS structure. We also show that these residues are primarily histidines. However, the exact histidines vary by ion, and the binding of metal ions disrupts active site integrity. As for the human GS filament, we show that assembly occurs under all substrate conditions. We also show that the human GS filament has the potential to turn over substrates and therefore, unlike the *E. coli* GS filament, is active.

Chapter 2: *E. coli* Glutamine Synthetase Filaments

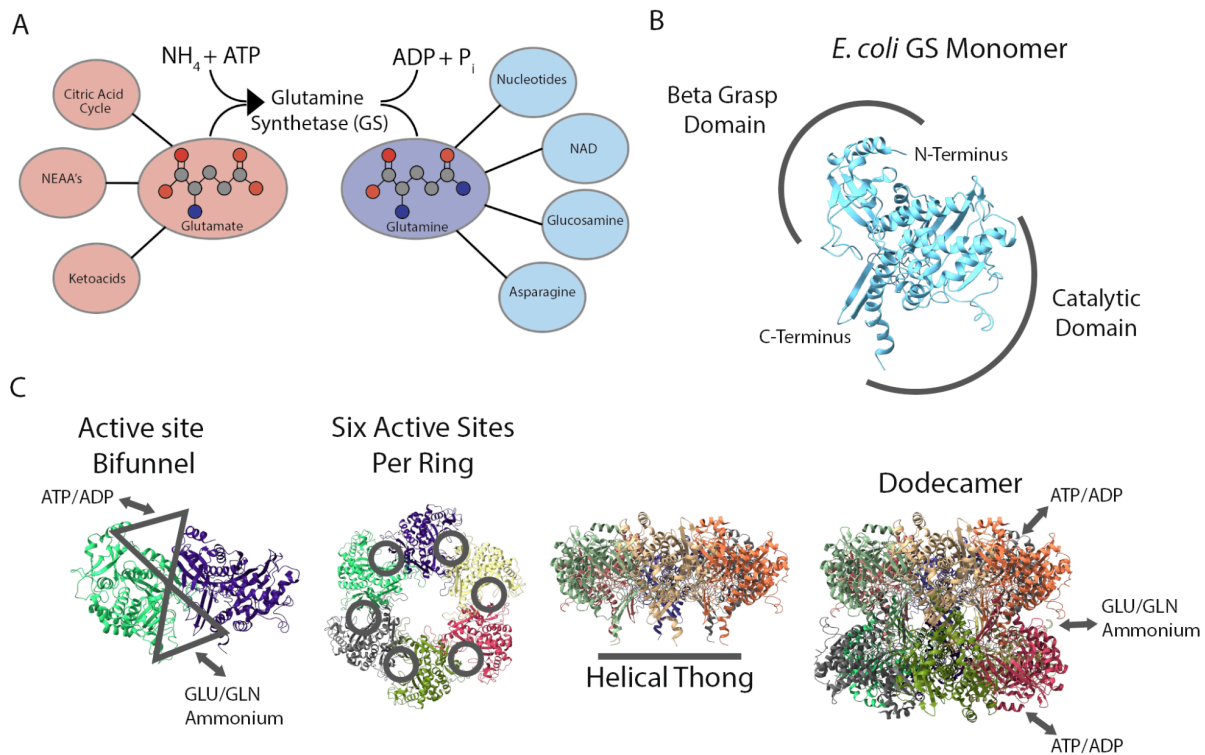


Figure 1: The Structure and Role of GS.

A) Metabolic pathways associated with glutamine-glutamate flux and the substrate/products of GS. B) *E. coli* GS monomer emphasizing the beta-grasp domain, catalytic domain, N-terminus and C-terminus. C) The oligomeric assembly of the functional *E. coli* GS dodecamer, the location of the catalytic bifunnel, the position of the active sites across the rings, the helical thong position, and the ligand cycling sites.

A) *E. coli* N-terminal Residues are Vital for GS Filament Formation

E. coli GS (residues 1-469), with an N-terminal 6xHis-SUMO tag, was expressed in BL21 RIL cells, then purified using IMAC, followed by tag removal with ULP1, and further purification with SEC. This process yielded *E. coli* GS (residues 1-469) with a single leading serine residue scar (Ser-GS) of appropriate molecular weight and minimal contaminants (**Figure 3A**). We examined Ser-GS under negative stain electron microscopy (EM) in the presence of multiple divalent cations and recapitulated filament formation under cobalt (Co) conditions (Valentine et al., 1968) (**Figure 3B**). To investigate the interface architecture and the effects of

filament formation on the structure of Ser-GS, we resolved a 2.6 Å and 2.8 Å cryo-EM structure of Ser-GS, with Co and Mg respectively (**Figure 2, 4**). We built an atomic model of the Co-induced filament interface and compared it to the published Ni filament structure (Huang et al., 2022). We observed significant differences between the Co and Ni interfaces, including an 8 Å separation between interface-forming H1 helices (**Figure 3D**) and the presence of multiple metal ions under Co at the interface.

Our structure shows the N-terminal residues of Ser-GS play a key role in metal ion coordination at the interface (**Figure 3C**). The leading serine residue of Ser-GS was unresolved in the cryo-EM map. It appears Met-1 and His-5 coordinate one of the Co ions with Met-1' and His-5' from the other interacting protomer (**Figure 3C**). The other metal ions are coordinated by Glu-12, His-13, Glu-12', His-13', and an extensive water network (**Figure 3C**). However, proteomics data reveal that the Met-1 of GS *in vivo* is post-translationally removed by methionine aminopeptidases (Hirel et al., 1989; Link et al., 1997). Therefore, scarless-GS (residues 2-469), with the leading serine and initial methionine removed from the expression construct, was used to investigate the native protein structure.

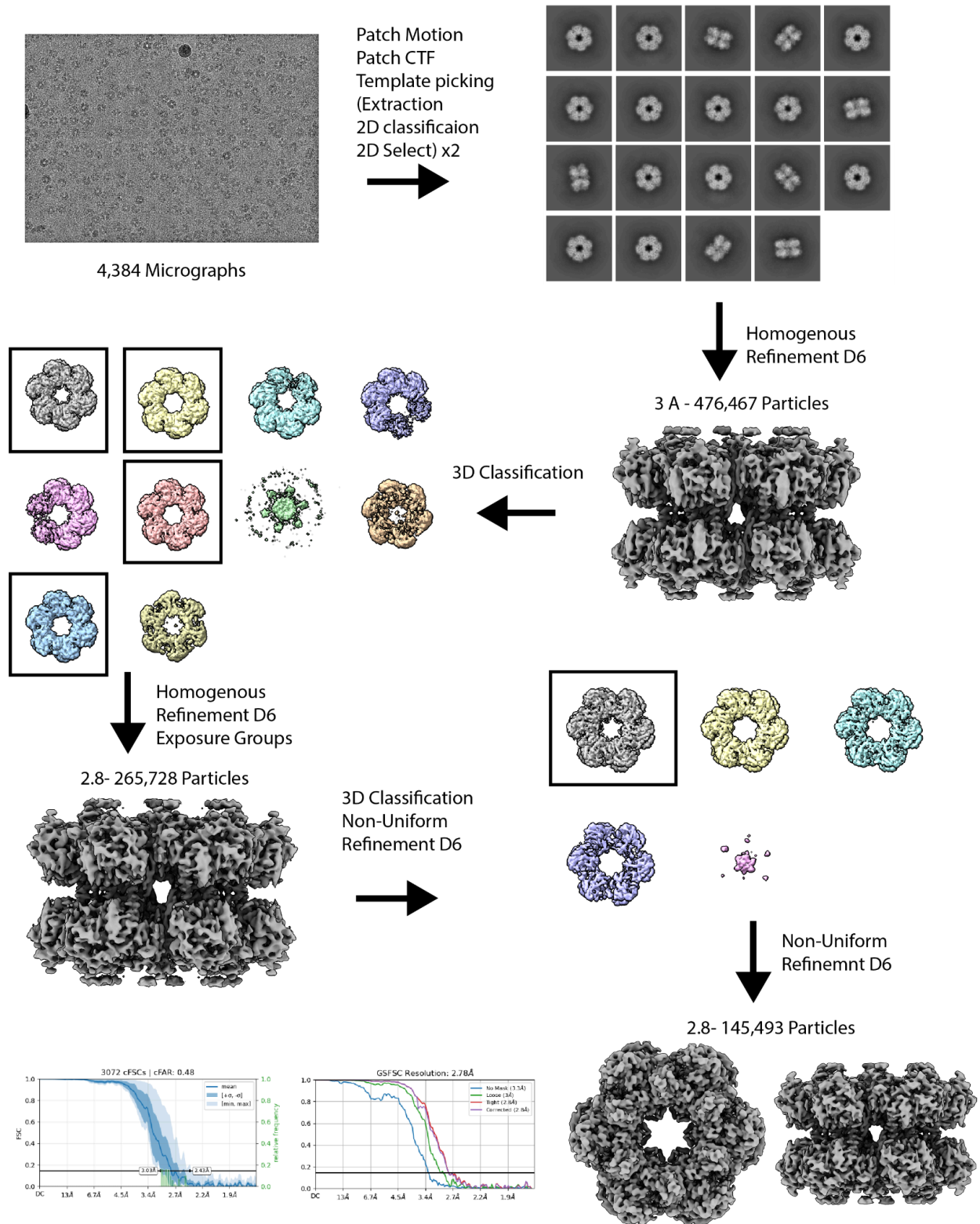


Figure 2: SM-GS Mg Processing.

Image processing workflow with an example micrograph, Gold Standard FSC and Conical FSC from CryoSPARC.

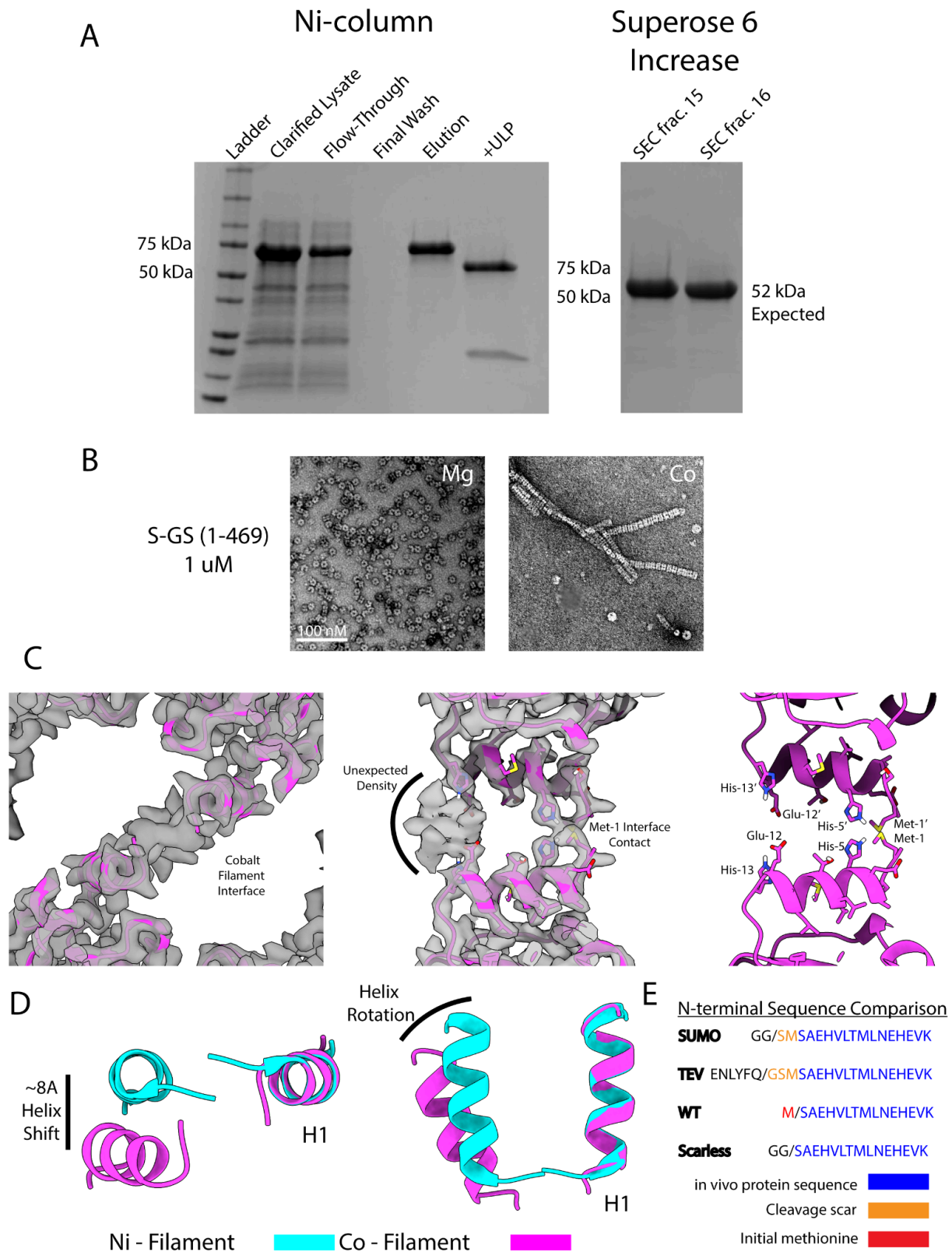


Figure 3: SM-GS Co and Ni Interface Comparison.

A) Denaturing protein gel of various Ni-Column and SEC fractions showing purification of *E. coli* GS at the expected molecular mass of 52kDa and removal of the 6xHis-SUMO tag after ULP addition. B) Dodecamer formation of purified *E. coli* SM-GS under Mg and filament formation of *E. coli* SM-GS under Co. C) Views of the *E. coli* SM-GS H1 interface structure with and without the electron potential map overlaid, illustrating the large unexpected density found between the H1 helices, the Met-1 interaction, and positioning of other residues composing the H1 helices of the interface forming protomers. D) Overlay of the experimentally produced Co H1 interface helices and the published Ni H1 interface (7W85) aligned at one helix labeled H1. E) Comparison of the N-terminal residues between the constructs used to investigate the filament interface and the *in-vivo* peptide sequence.

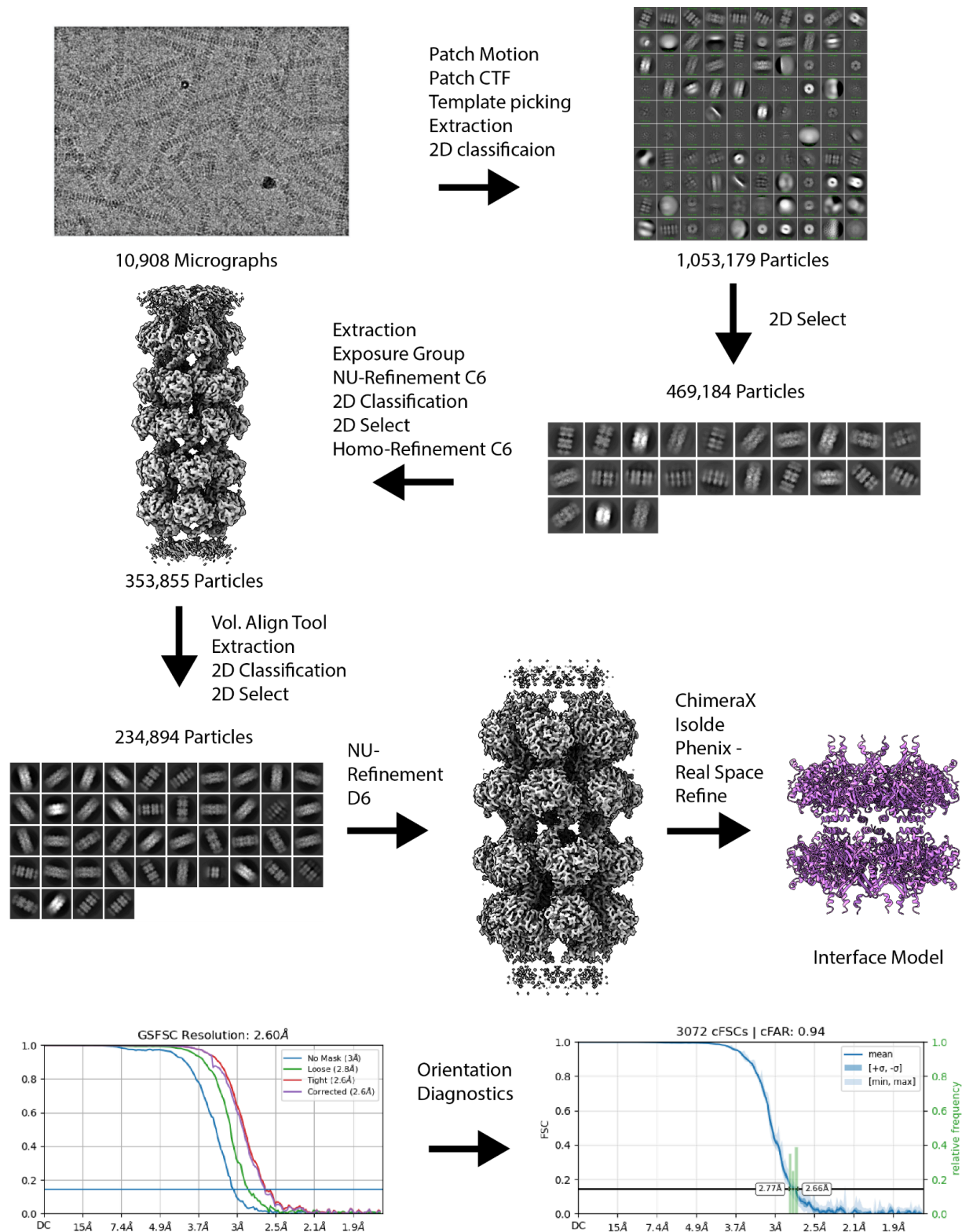


Figure 4: *E. coli* SM-GS Co Processing.

Data processing workflow with an example micrograph, Gold Standard FSC and Conical FSC from CryoSPARC.

B) Scarless-GS Filaments Reveal a Novel Filament Interface Architecture (Binuclear)

Scarless-GS under negative stain EM followed a similar pattern of assembly previously observed by Ser-GS (**Figure 5A**). Filaments were readily observed under 1 mM Co, Ni, or Zn conditions after 30 minutes. At concentrations higher than 1 mM, filaments would assemble into larger bundles. At lower concentrations, neither filament assembly nor bundling occurred with Co, and minimal dodecamer stacking occurred with Ni. Incubation for up to 72 hours with 0.1 mM Co or Ni ions failed to produce any further assembly, indicating filaments are metal ion concentration-dependent (**Figure 5C**). Unlike Ni and Co, Zn induced filaments at 50 μ M alone and as low as 10 μ M when dodecamer assembly was first stabilized with 10 mM of Mg. Visually, scarless-GS filaments appeared similar to Ser-GS filaments at the limited resolution of negative stain EM and supported the head-to-head assembly of dodecamers in the greater filament. (**Figure 1B, 5A**).

We resolved a 2.3 Å structure of scarless-GS under 1 mM Co. Scarless-GS revealed new architecture between the H1 helices of interface-forming protomers. In the scarless-GS Co structure (with or without 10 mM Mg) there was a 12 Å compaction of the interface compared to Ser-GS. The large ion density previously seen in the Ser-GS model was no longer visible, supporting the idea that the leading SM residues were coordinating assembly in a non-native manner (**Figure 1C**). Additionally, the H1 helices were repositioned to be in closer contact. Importantly, the scarless-GS Co structure showed that His-5 and His-13 of interacting protomers were still involved in coordinating the divalent cations at the interface (**Figure 5D**). This highlights the importance of these residues in assembly. However, unlike the published Ni structure (Huang et al., 2022), the Co interface appears binuclear, with 2 metal ions coordinating assembly with the assistance of two water molecules. Histidines at position 5 each coordinate a Co ion through interaction with the epsilon nitrogen, while the histidines at position 13 work together to coordinate two water molecules, again with their respective epsilon nitrogen. To note, the interface requires the coordinating histidines to be in the delta tautomer state, where the delta nitrogen is protonated. The structures were collected at pH 8, where histidine resonates between the epsilon and delta protonation states. This may indicate that for filament formation to occur, Co must bind to the delta tautomer histidine.

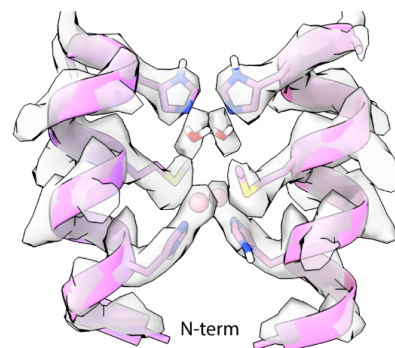
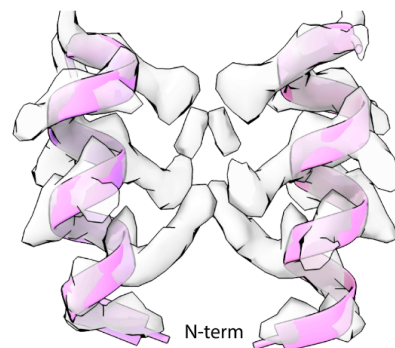
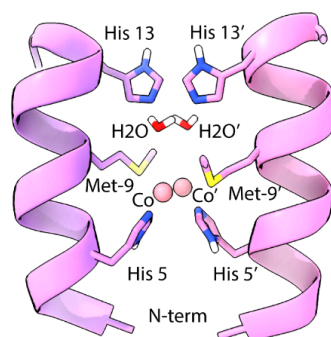
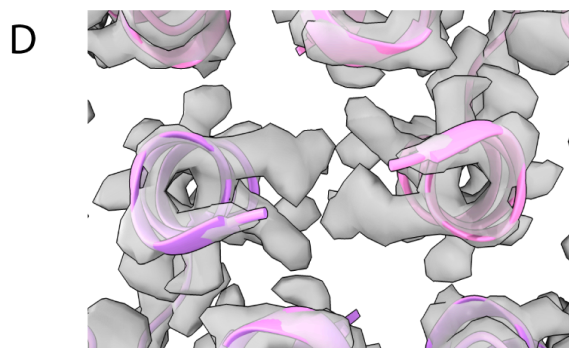
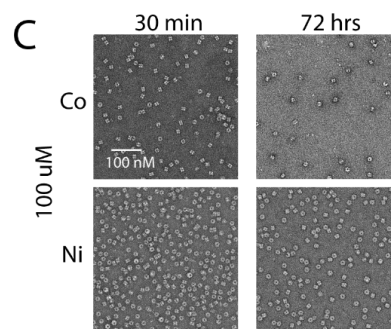
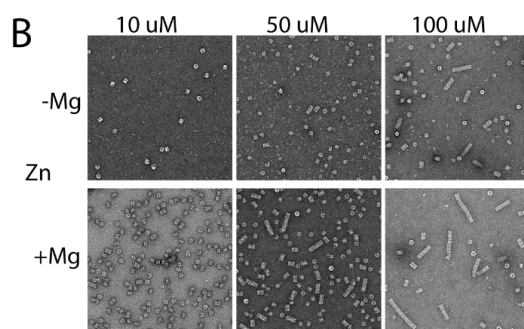
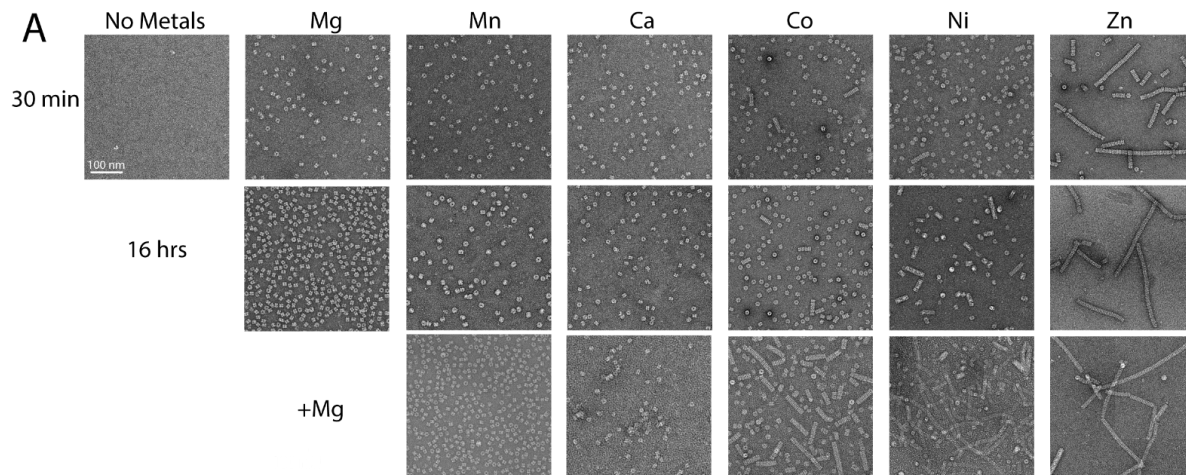


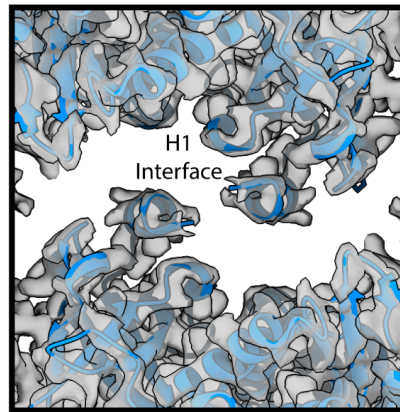
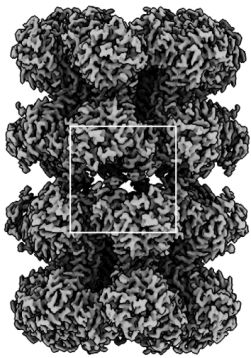
Figure 5: Scarless *E. coli* GS assembly and the Scarless Co-only Interface.

A) Assembly of scarless *E. coli* GS with or without 1 mM metal supplementation for 30 minutes, 16 hours, or 30 minutes with 10 mM Mg. B) Titration of scarless *E. coli* GS under Zn with or without 10 mM Mg. C) Incubation of scarless *E. coli* GS for 30 minutes or 72 hours with 100 μ M Co or Ni. D) Views of the scarless *E. coli* GS H1 interface structure with and without the electron potential map overlaid illustrating residue position and metal association.

C) Scarless-GS Ni Structure Reveals Novel His-37 Interaction

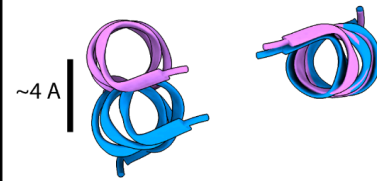
The binuclear interface identified under scarless-GS Co conditions led us to reinvestigate the Ni-induced filament structure using the scarless protein construct. A cryo-EM structure of scarless-GS with Ni was resolved to a resolution of 2.5 Å (**Figure 7A**). Again, the H1 helices were shown to coordinate filament assembly. The position of the H1 helices was similar to our Co structure, however, when aligned to a single interface helix, a ~4 Å extension of the interface was noted in the Ni structure (**Figure 7B**). Additionally, the density of the H1 helices in the Ni condition was less well-defined than the Co structure, with a particularly weak density near His-13 (**Figure 7C**). Lack of resolution indicates that the H1 helix is flexible, even when forming the interface, and this flexibility may explain why the identity of the metal ion present influences the filament interface architecture (**Figure 5D, 7C**). In an attempt to improve the density at the interface, particles were D6 symmetry expanded, and a focused refinement was performed with masking around two protomers participating in the H1 interface contact (**Figure 8**). While focused refinement failed to improve the density between H1 helices, it uncovered a new minor interface contact located between two H1 helical pairs of the major contact (**Figure 7D, E, F**). Masking across four protomers was used to resolve this new interface (**Figure 8**). It was revealed to be a symmetric contact formed between the H37s of each protomer (**Figure 7D**). The H37 pair appears to be coordinating a Ni ion with the assistance of ordered waters (**Figure 7E, F**). However, this is not the same protomer pair participating in the H1 helices contact. Instead, a single protomer from one decamer interacts with two adjacent protomers from the other decamer to form each contact (**Figure 7D**). This contact did not appear within the Co structure, even after focused refinement. Additionally, the H37s of the Co structure are in an orientation that does not support metal binding in that area (**Figure 7F**). Even when placed in the same orientation as the Ni H37 residues it appears that they would be unable to facilitate a metal ion between them without clashing. These observations indicate that the H37 contact may be unique to Ni-induced filament assembly, which is facilitated by the interface extension noted previously (**Figure 7B**). However, the H37 contact interface is only present in 18 percent of the symmetry-expanded and locally refined particles (**Figure 20**). A majority of the H37 residues adopt the Co orientation and are not involved in the interface (**Figure 20**). This suggests that it is not required for filament assembly and therefore is labeled as a minor contact.

A Scarless-GS Ni
Filament Map 2.4 Å

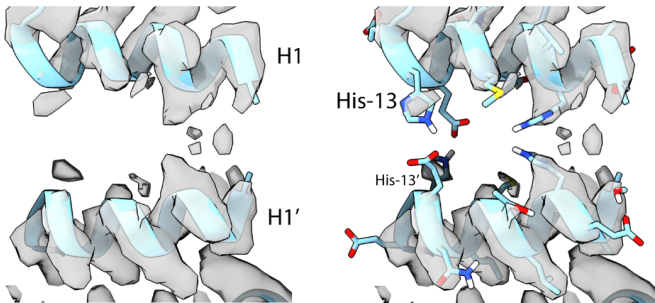


B

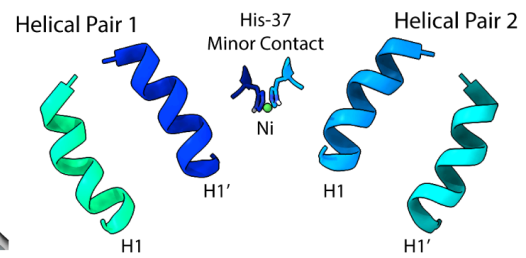
H1 Helices
Co vs Ni



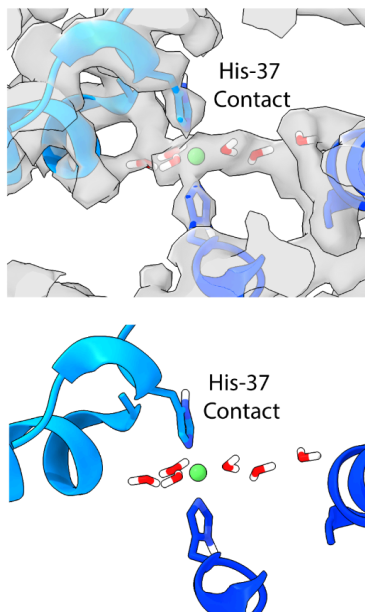
C



D



E



F

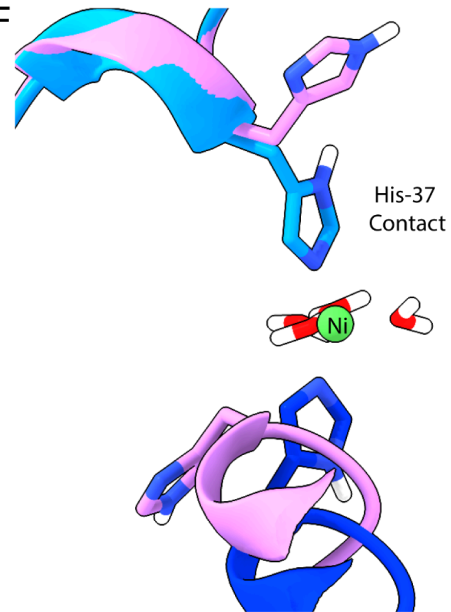


Figure 7: Scarless *E. coli* Ni Interface and His-37 Contact.

A) The scarless *E. coli* GS filament map with a window showing a zoom-in of the H1 interface with the model fit into the density. B) Comparison between the position of the scarless Co H1 interface (lavender) and Ni H1 interface (aqua) after alignment on one interface helix. C) The Ni H1 interface with and without residues, specifically around His-13. D) Model showing the location and protomers forming the minor H37 interface. E) Density and model for the H37 interaction with Ni (green) and waters. F) An overlay of the H37 loops showing the differences in residue orientation between Co filaments (lavender) and Ni filaments (aqua).

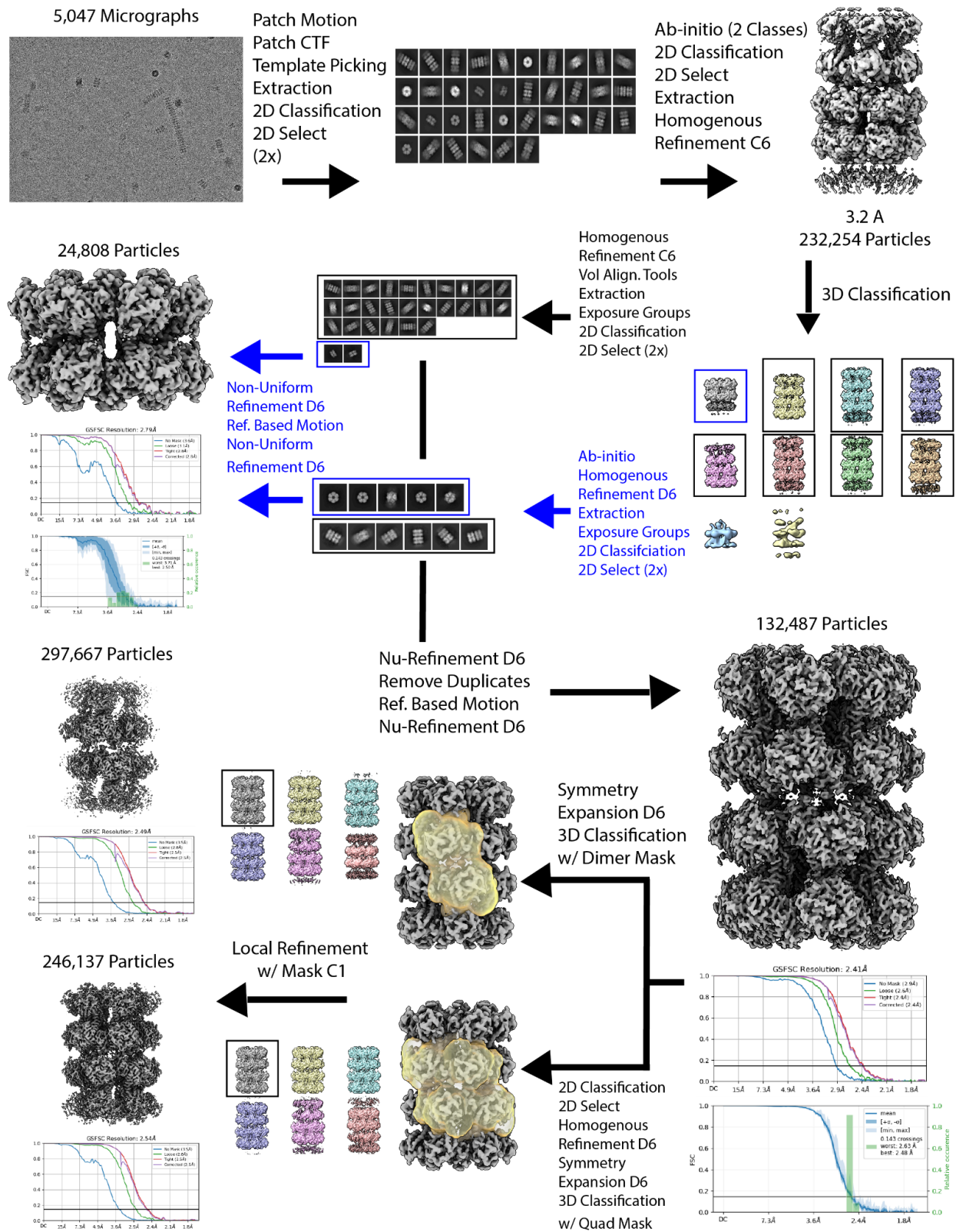


Figure 8: Scarless-GS Ni Processing.
Data processing workflow with an example micrograph, Gold Standard FSC and Conical FSC from CryoSPARC.

D) Zn Filaments Show Cations Interacting with H13

Zn promoted filament formation under the lowest ion concentration (**Figure 5B**). A cryo-EM structure of scarless GS with Zn was resolved to 2.3 Å to determine if the architecture at the interface could explain this observation (**Figure 9A, 11**). Overall, when aligned to a single H1 helix, the position of the H1 helices at the interface more closely resembles the Ni filament, however with a 1-2 Å increased separation between the H1 and H1' helices (**Figure 9B, E**). At least two Zn ions were shown to be bound at the H1 helix interface. They are coordinated by histidine-13 and glutamate-12 from both interacting protomers. The involvement of Glu-12 at the interface was not directly observed in the Co or Ni filament (**Figure 5D, 7C**).

Additionally, density supported the presence of two water molecules interacting with the Zn ions. Interestingly, there was little density to support a rigid interface interaction that depended on histidine-5 (**Figure 9C**). Only when contour levels were adjusted did the map reveal an interaction between histidine-5 pairs (**Figure 9D**). This H13 dependence appears unique to the Zn structure. At the Co interface, the two metal ions are interacting with histidine-5, and two waters appear to be coordinated with the H13s, while in the Ni filament, the H13 residues are unresolved (**Figure 5D, 7C**). Additionally, the minor H37 interaction observed under Ni conditions was not seen in the Zn filament. Histidine 37 under Zn conditions adopted the same confirmation as the Co filament (**Figure 9F**). When aligned to one protomer a ~3 Å shift is seen in the position of the H37 loop (**Figure 9F**). This separation is likely a propagation from the increased separation of the H1 helices noted previously (**Figure 9B**). This shift prevents the minor interaction from forming under the Zn filament conditions because the H37s are too far away to sequester a cation.

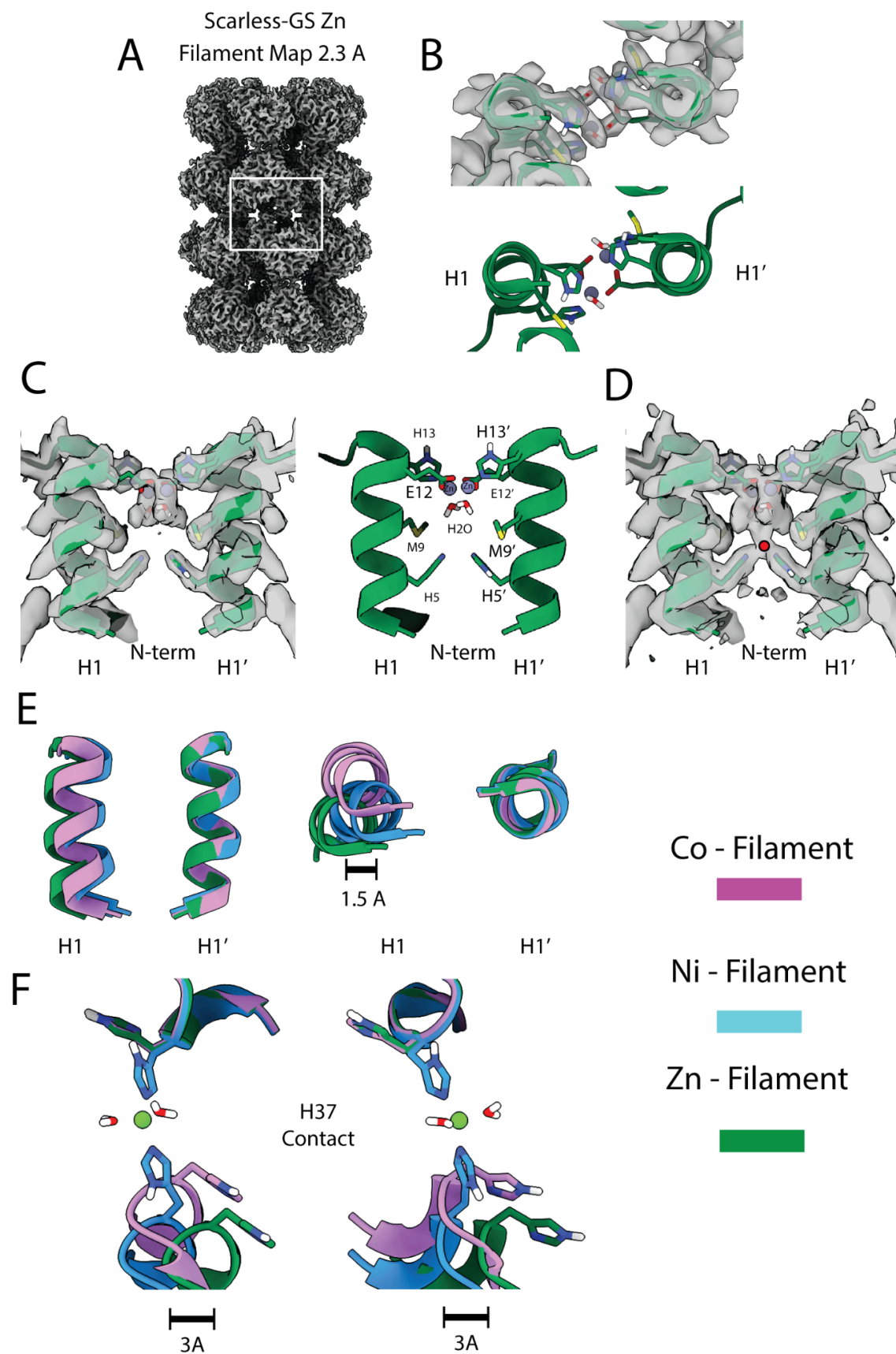


Figure 9: Scarless *E. coli* Zn Interface and Co, Ni, Zn Interface Comparison.

A) Zn filament map with a window emphasizing the interface. B) Side view of the Zn H1 interface with residues and Zn ions (purple) with or without the map overlaid. C) Transverse view of the filament interface showing the position of Zn (purple) ions and waters near H13 with or without map overlaid. D) Transverse of the Zn H1 interface at a lower threshold than C) to show weak density around H5. E) Overlay of the Co (lavender), Ni (aqua), and Zn (moss) ribbon models at the H1 interface after alignment on H1' showing a 1.5 Å shift in the Zn H1 compared to Ni. F) Overlay of the Co (lavender), Ni (aqua), and Zn (moss) ribbon models at the H37 loop with Ni (green) and waters. Alignment was done on one loop to show the 3 Å lateral shift in the Zn 37 loop compared to Ni.

E) Ni and Zn ions cause the loss of active site integrity

Besides the structural differences at the interface of Co, Ni, and Zn-induced filaments, Ni and Zn cations caused direct changes to the active site residues of glutamine synthetase. One major change is the loss of density corresponding to residues 154-190 in the Ni and Zn *E. coli* GS structures (**Figure 10C**). The active site of GS is composed of residues from two adjacent protomers in each hexameric ring (**Figure 1C**). Residues 154-190 are ordered loops within this pocket and extend into the lumen of the rings (**Figure 10A, B**). These residues form the ammonia binding pocket within the active site and contain various residues conserved among prokaryotes, eukaryotes, or all organisms (**Figure 10J**) (Eisenberg et al., 2000). Our Ni and Zn filament structures reveal that a single cation disrupts the proper folding of this region of scarless-GS by binding to H212 from one protomer and H31 of the neighboring protomer (**Figure 10E, G**). This coordination repositions the loop containing residues 211-219 compared to the Co-induced filament and Mg dodecamer structures (**Figure 10D, F, H, I**). The new placement of the 211-219 loop directly clashes with the packing position of residues within the 154-190 loops containing primarily aromatic and hydrophobic residues (**Figure 10D, I, M**). The loss of the hydrophobic packing in this area most likely results in the complete disorder of the 154-190 loops.

Additionally, the 211-219 loop shift in the Ni and Zn filament structures also impacts one of the metal-binding sites within the active site. Glutamate 132, 213, and 221 coordinate a divalent ion responsible for stabilizing the glutamine synthetase dodecamer and binding the γ -carboxylate group of glutamate, which receives the γ -phosphate from ATP and forms the intermediate. The 211-219 loop shift repositions E213 away from E132 and E221. This results in H211 coordinating the divalent ion with E213 as opposed to E132 and E221 (**Figure 10K, L, M**). The new H211 and E213 metal coordination moves the divalent ion 3 Å away from its known binding position (**Figure 10M**). The rearrangement of the glutamate binding site and the loss of the ammonium bind site coordinated by Tyrosine-180 suggest ligation of ammonia to glutamate to form glutamine is probably lost under Zn and Ni-only filament conditions. Mg is known to be bound to *E. coli* GS *in vivo* within the active site for catalysis and dodecamer stabilization (Abell et al., 1991). To investigate whether Zn and Ni disrupt the active site under this condition cryo-em structures of Mg stabilized dodecamers that were then supplemented with Co, Ni, or Zn to induce filaments were resolved to 2.3, 2.4, and 2.4 Å respectively (**Figure 13,14,15**). All three structures showed loss of the 154-190 loops and disruption of the glutamate binding site. (**Figure 19A**). The ATP binding site appears unaffected by Co, Ni, and Zn with or without Mg (**Figure 19B**).

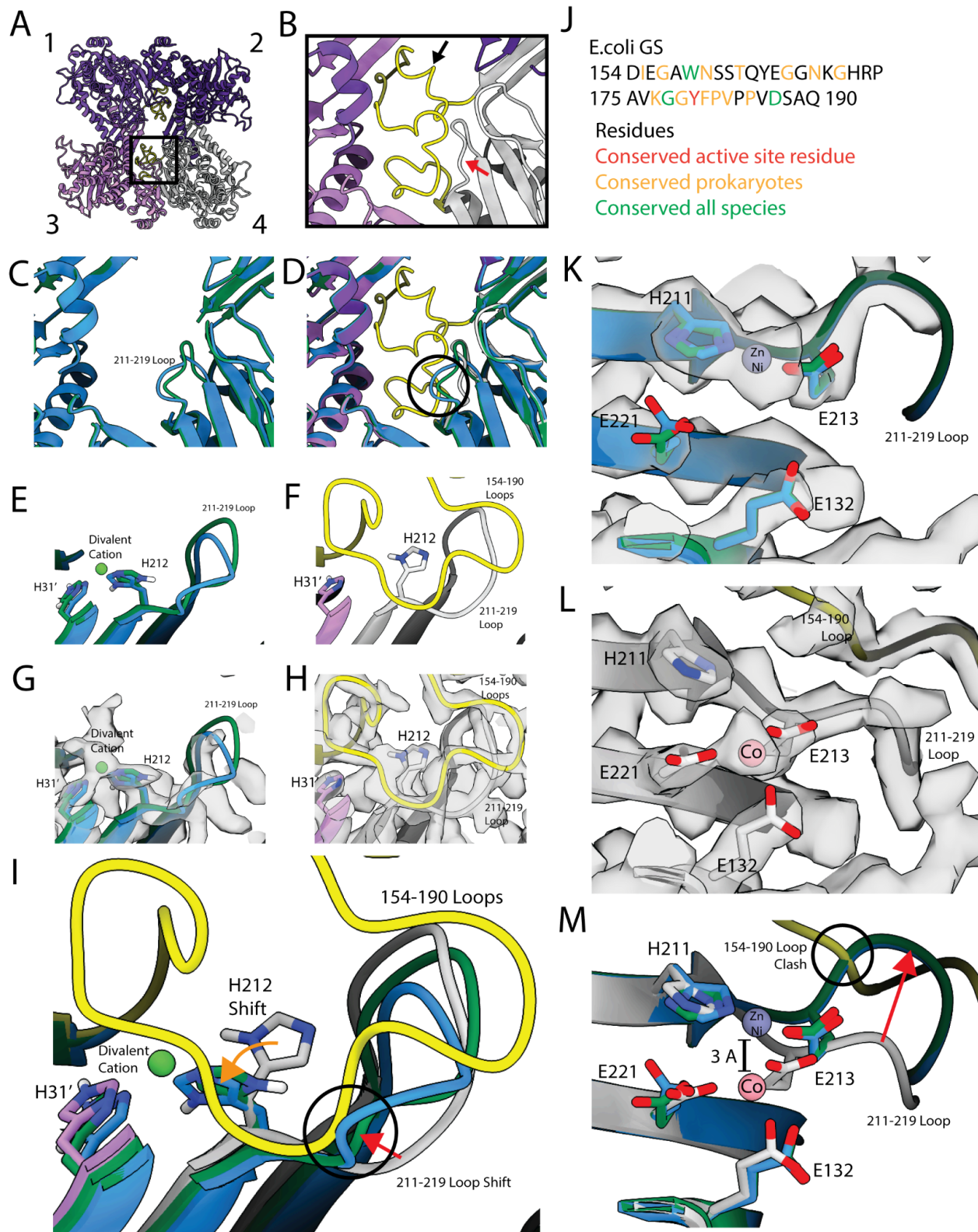


Figure 10: *E. coli* Active Site Integrity Changes Under Metals.

A) Ribbon structure of 4 protomers of a Co filament dodecamer coming together to form two active sites with residues 154-190 (yellow). B) Zoom in of the window in 10A emphasizing residues 154-190 (yellow, black arrow) and the 211-219 loop (red arrow). C) Ribbon overlay of Ni (aqua) and Zn (moss) filament dodecamer 154-190 residue region with the 211-219 loops labeled. D) Ribbon overlay of the Co (lavender), Ni (aqua), and Zn (moss) 154-190 Loops region showing the clash (black circle) between the Co 154-190 residues (yellow) and Ni/Zn 211-219 loops. E) Overlay of residues H212 and H31' coordinating a divalent cation in the Ni (aqua) and Zn (moss) dodecamer. F) Position and orientation of H212 and H31' in the Co filament dodecamer. G) E with Zn filament map overlay. H) F with the Co filament map overlay. I) Overlay of the Co (lavender/white), Ni (Aqua), and Zn (Moss) H212 and H31' residue differences (orange arrow) with Co 154-190 (yellow) and 211-219 loops labeled. 211-219 loop shifts in Ni and Zn compared to Co are indicated (red arrow). The clash between 211-219 loop in Ni and Zn filaments with residues 154-190 in Co is noted (black circle). J) Conservation of *E. coli* residues 154-190 in GS. K) H211 and E213 coordinating a metal ion in the Ni filament dodecamer (aqua) and Zn filament dodecamer (moss) with the Zn filament map overlaid and loop 211-219 labeled. L) E132, E213, and E221 in the Co filament dodecamer coordinating a Co ion with the Co filament map overlaid and residues 154-190 (yellow) and 211-219 labeled. M) Overlay of E132, E132, E213 and H211 of the Co (white), Ni (aqua), and Zn (moss) filament dodecamer showing the 3 Å ion shift and the 211-219 loop shift (red arrow) leading to the clash with residues 154-190 (black circle)

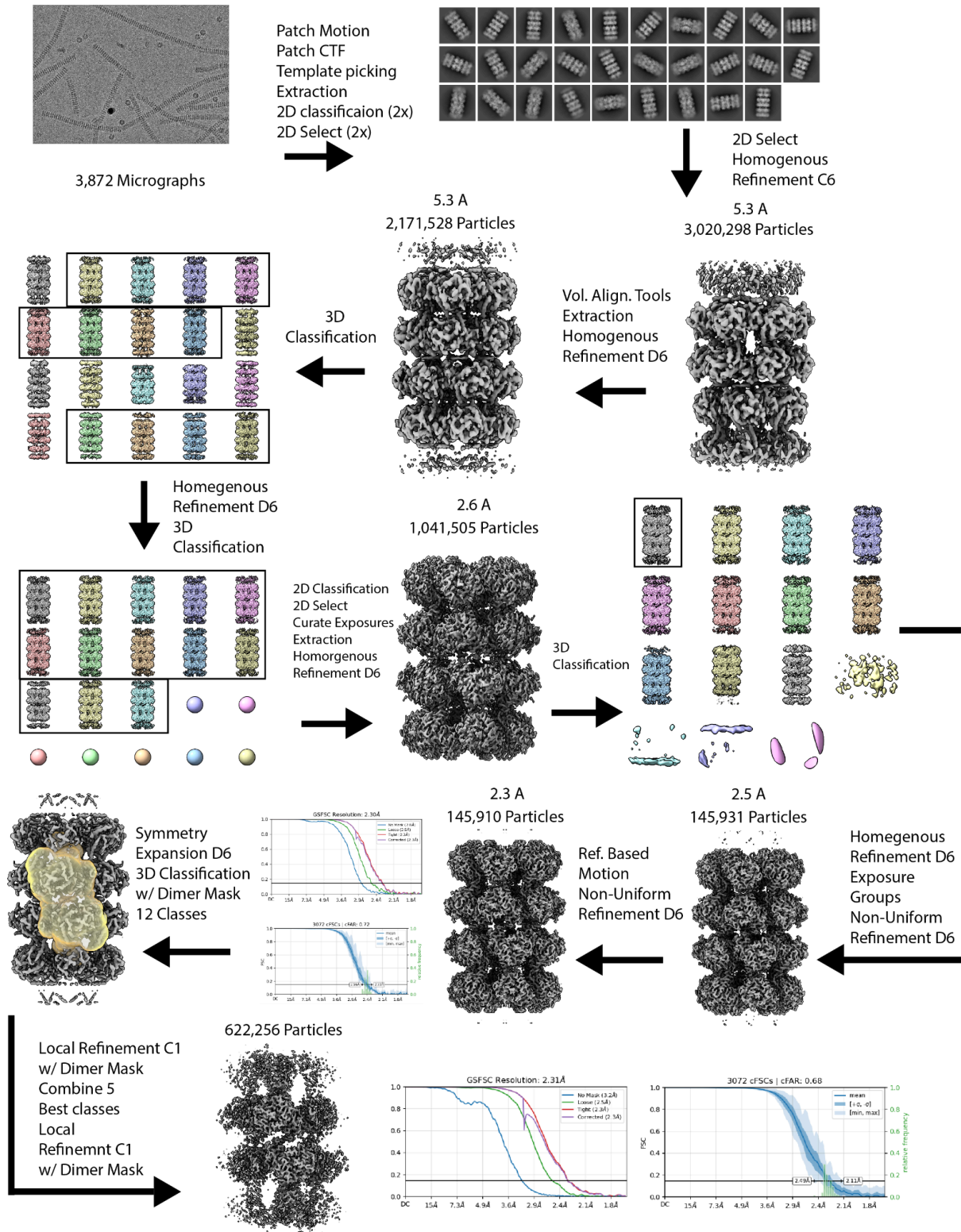


Figure 11: Scarless-GS Zn Processing.

Data processing workflow with an example micrograph, Gold Standard FSC and Conical FSC from CryoSPARC.

F) Histidine, pH, and substrates impact filament assembly

Interface mutants H5A, H13A, and H37A were cloned and purified to test their effect on filament assembly using negative stain. H37A, the minor interface mutation, did not appear to influence filament formation (**Figure 12A**). This result supports the idea that filament assembly is dominated by the H1 interaction and the H37 contact is not required for robust assembly. The H5A and H13A mutants caused the complete collapse of filaments under Co or Ni conditions (**Figure 12A**). However, this was not the case for Zn-induced filaments (**Figure 12A**). The H5A mutation only partially disrupted filament formation under Zn. While H13A disrupted long filaments, some small dodecamer stacking is still observed.

Based on our structures, various histidine residues play a large role in filament formation and metal binding within the active site indicating pH may influence assembly. Lowering the pH of the assembly buffer conditions from pH 8 to pH 6 was shown to disrupt filament assembly (**Figure 12C**). When the pH was further lowered to pH 5, no assembly was observed, even in the presence of Mg for dodecamer stabilization (**Figure 12C**). The disruption of the ligand binding sites under Co, Ni, and Zn in the presence of Mg also led us to investigate the effect of substrates on filament assembly. Saturating levels of ATP, as exist for most metabolites in vivo (Bennett et al., 2009), completely ablated filament formation, while glutamate limited assembly (**Figure 12B**). Interestingly, ammonium did not affect filament assembly. To identify any structural changes after filament collapse, cryo-EM samples under Mg, Co, and ATP or Mg, Co, and Glutamate were collected and resolved to 2.8 and 2.1 Å, respectively (**Figure 16, 17**). Under ATP and glutamate, no filaments were observed in cryo-em, supporting substrate addition leads to filament collapse (**Figure 16,17**). Additionally, the active site and 154-190 loops matched their native state. Glutamate was found in its binding pocket with its carboxyl group associated with the metal ion coordinated by E213, E221, and E132 (**Figure 12D**). In the ATP sample, we found ADP bound to the active site, GS aberrant ATP hydrolysis (**Figure 12E**).

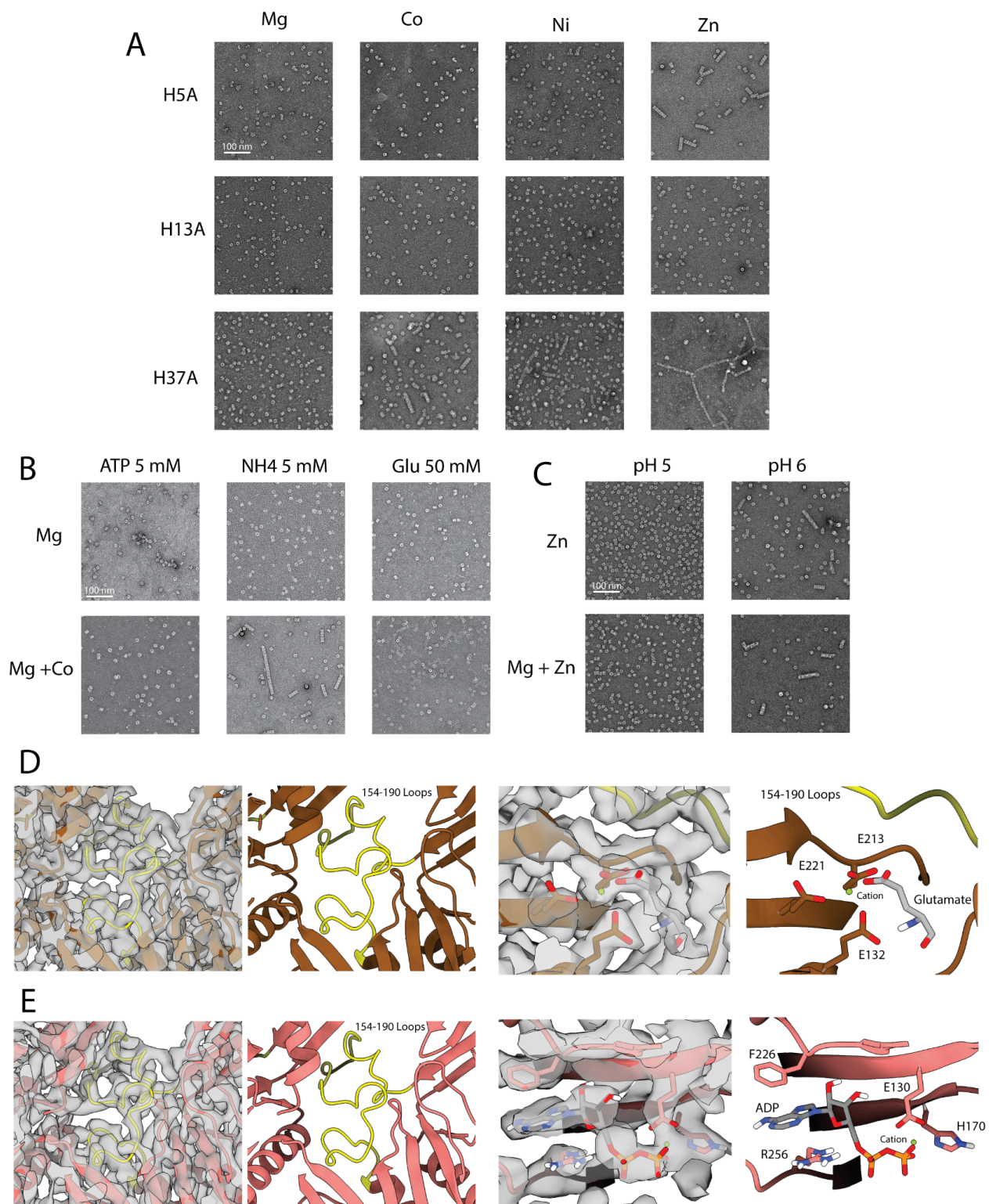


Figure 12: Breaking *E. coli* GS Filaments.

A) Negative Stain of scarless *E. coli* GS H5A, H13A, and H37A mutants with 1 mM of various metals. B) Negative stain of scarless *E. coli* GS under saturating substrate concentrations and 10 mM of Mg with or without 1 mM of Co. C) Negative stain of Scarless *E. coli* GS at pH 5 or Ph 6 and 1mM Zn with or without 10 mM Mg. D) The model of scarless *E. coli* GS (mocha), under 10 mM Mg, 1 mM Co, and 50 mM glutamate, with or without the map overlaid, of the 154-190 region (yellow, left) and glutamate binding site (grey, right). E) The model of scarless *E. coli* GS (salmon), under 10 mM Mg, 1 mM Co, and 5 mM ATP, with or without the map overlaid, of the 154-190 region (yellow, left) and ATP binding site (grey, right).

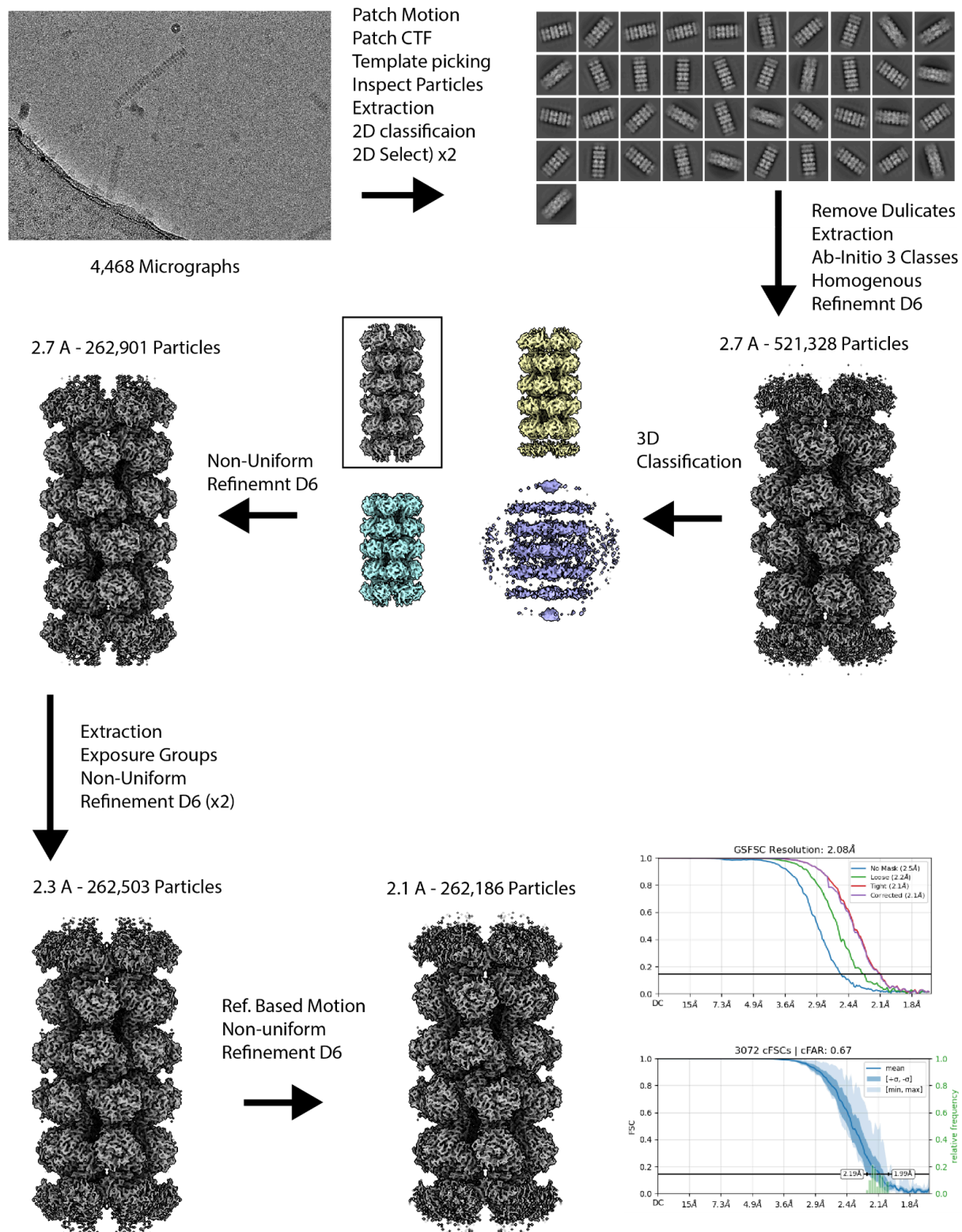


Figure 13: Scarless-GS Co w/ Mg Processing.

Data processing workflow with an example micrograph, Gold Standard FSC and Conical FSC from CryoSPARC.

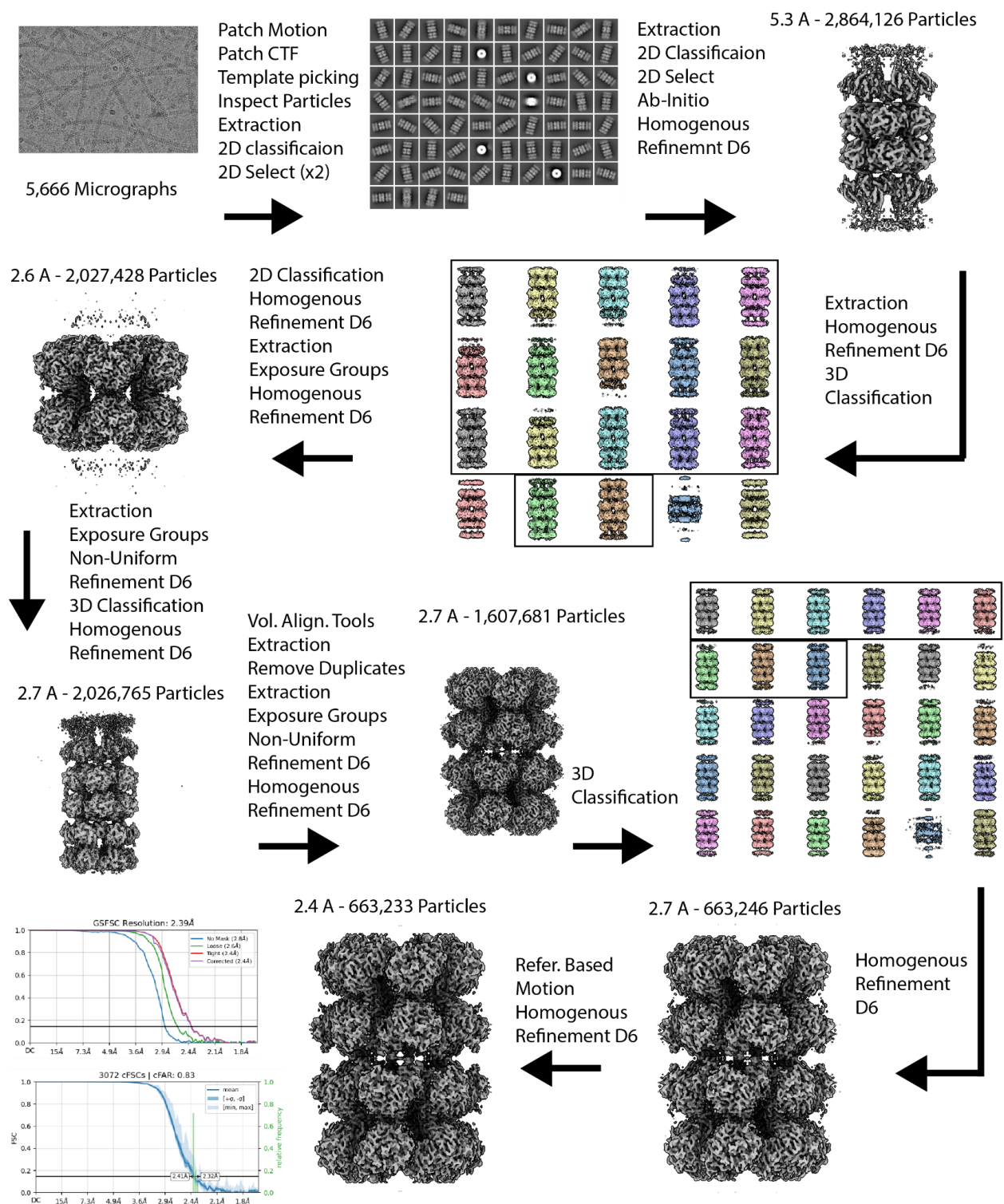


Figure 14: Scarless-GS Ni w/ Mg Processing.

Data processing workflow with an example micrograph, Gold Standard FSC and Conical FSC from CryoSPARC.

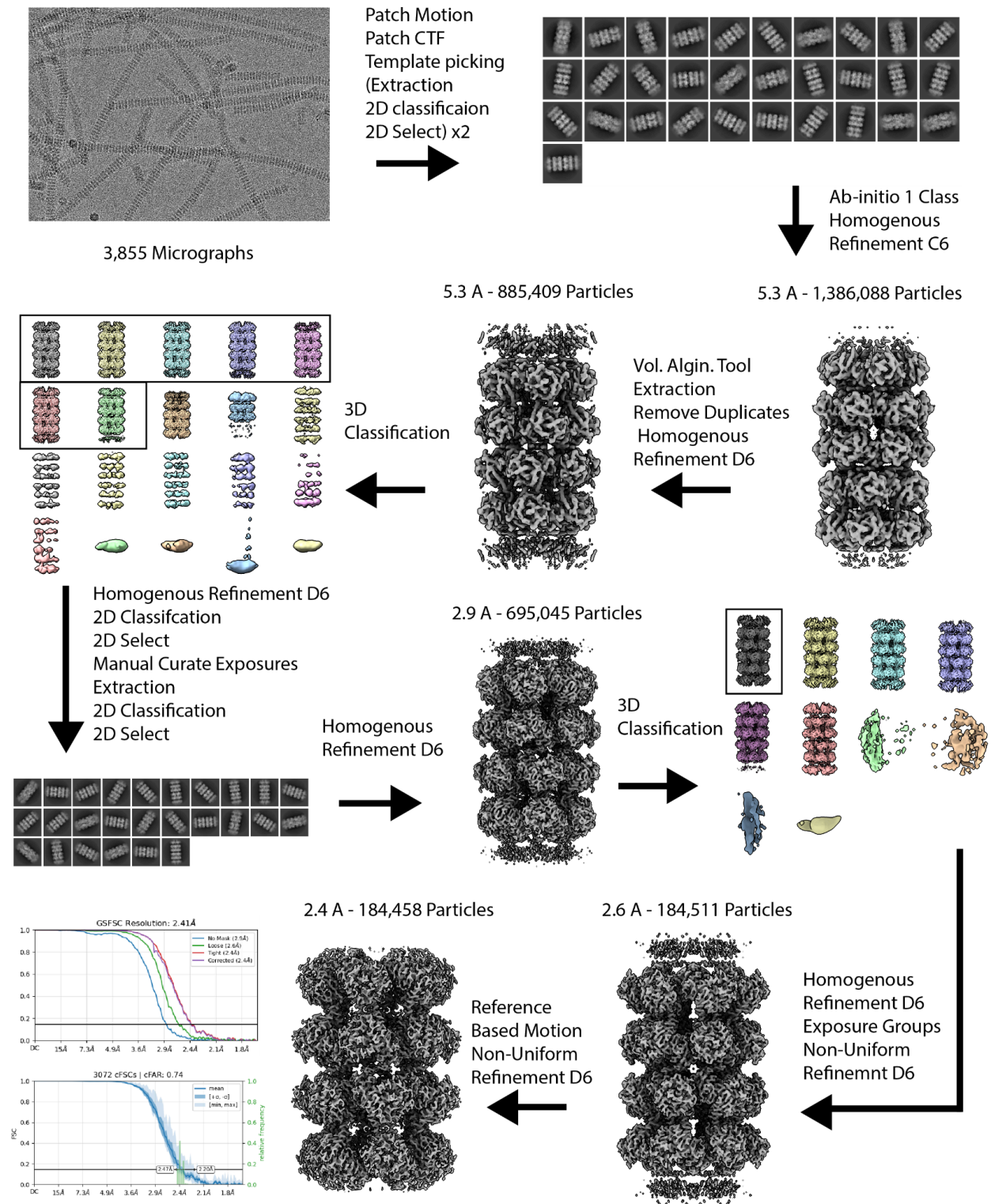


Figure 15: Scarless-GS Zn w/ Mg Processing.

Data processing workflow with an example micrograph, Gold Standard FSC and Conical FSC from CryoSPARC.

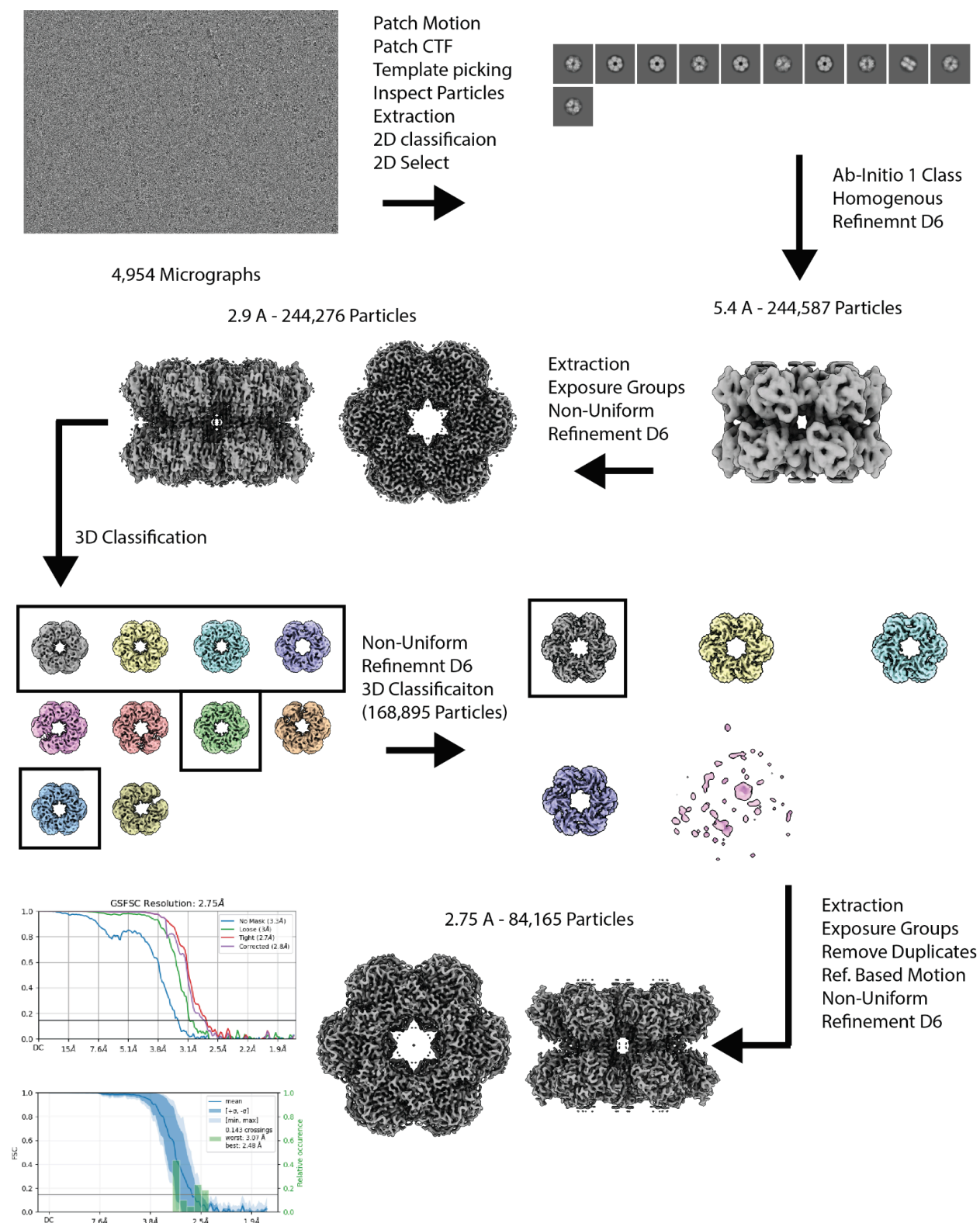


Figure 16: Scarless-GS with Co, Mg, and ATP Processing.

Data processing workflow with an example micrograph, Gold Standard FSC and Conical FSC from CryoSPARC.

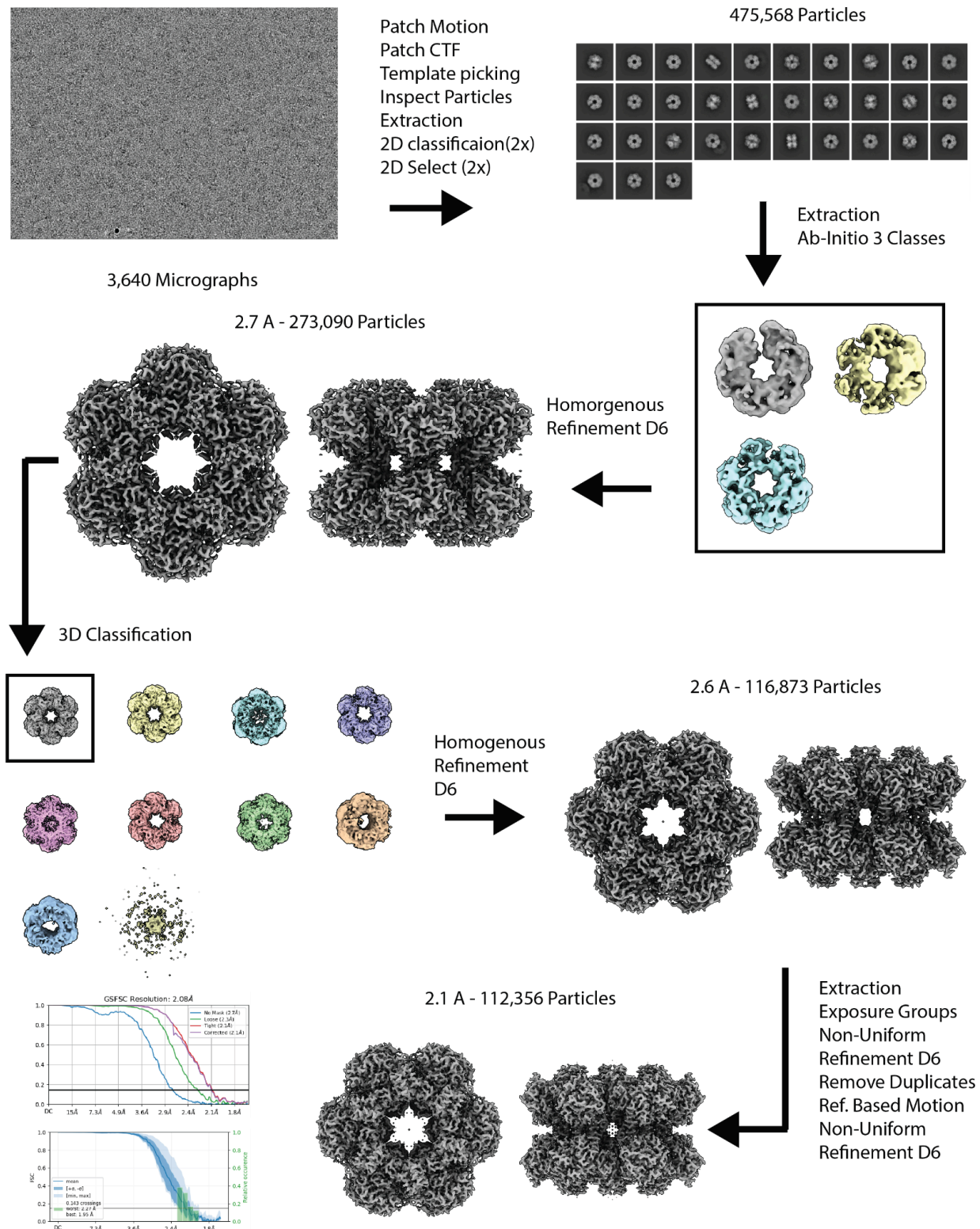


Figure 17: Scarless-GS with Co, Mg, and Glutamate.

Data processing workflow with an example micrograph, Gold Standard FSC and Conical FSC from CryoSPARC.

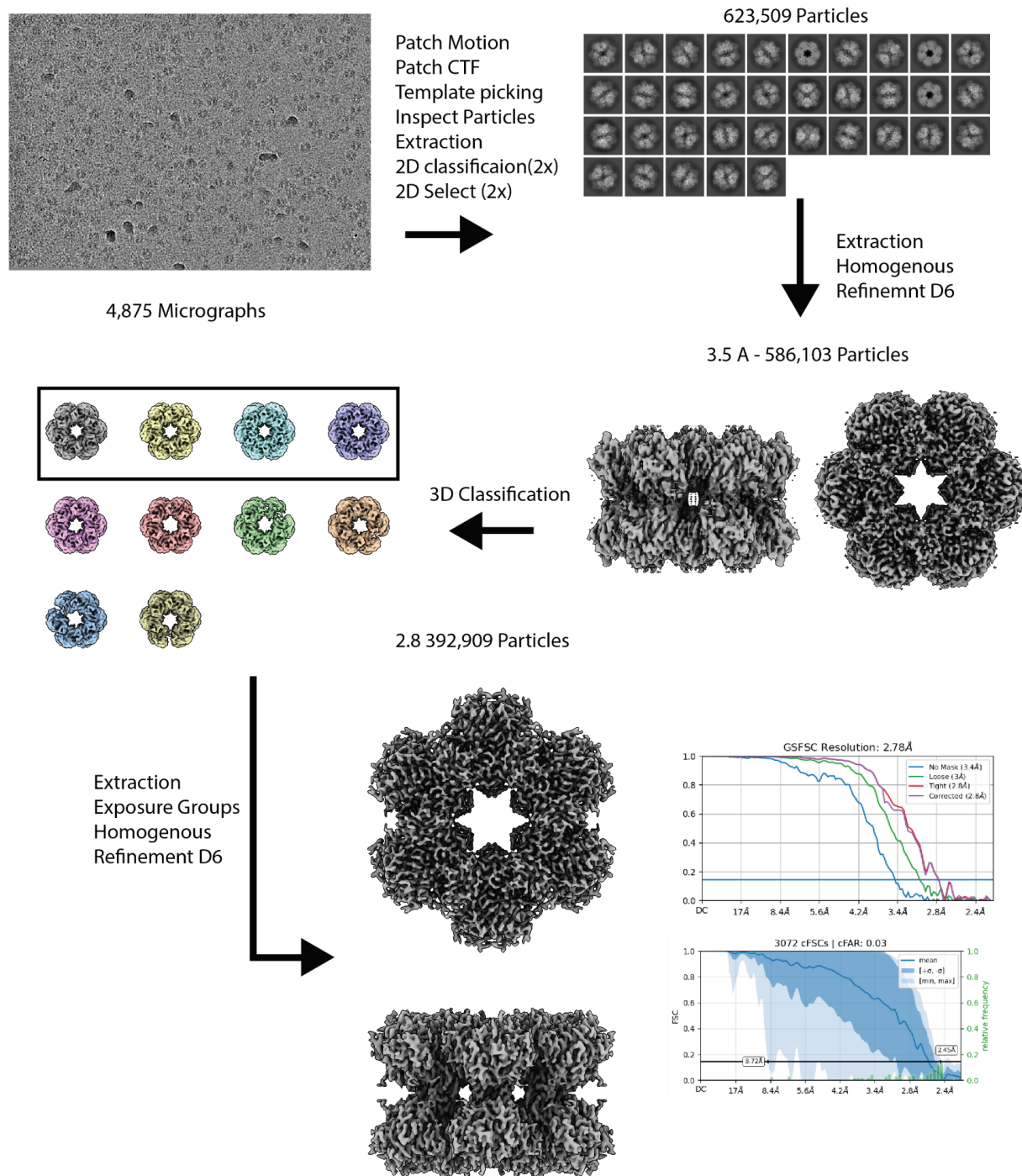


Figure 18: Scarless-GS Mg with Substrate Processing.

Data processing workflow with an example micrograph, Gold Standard FSC and Conical FSC from CryoSPARC.

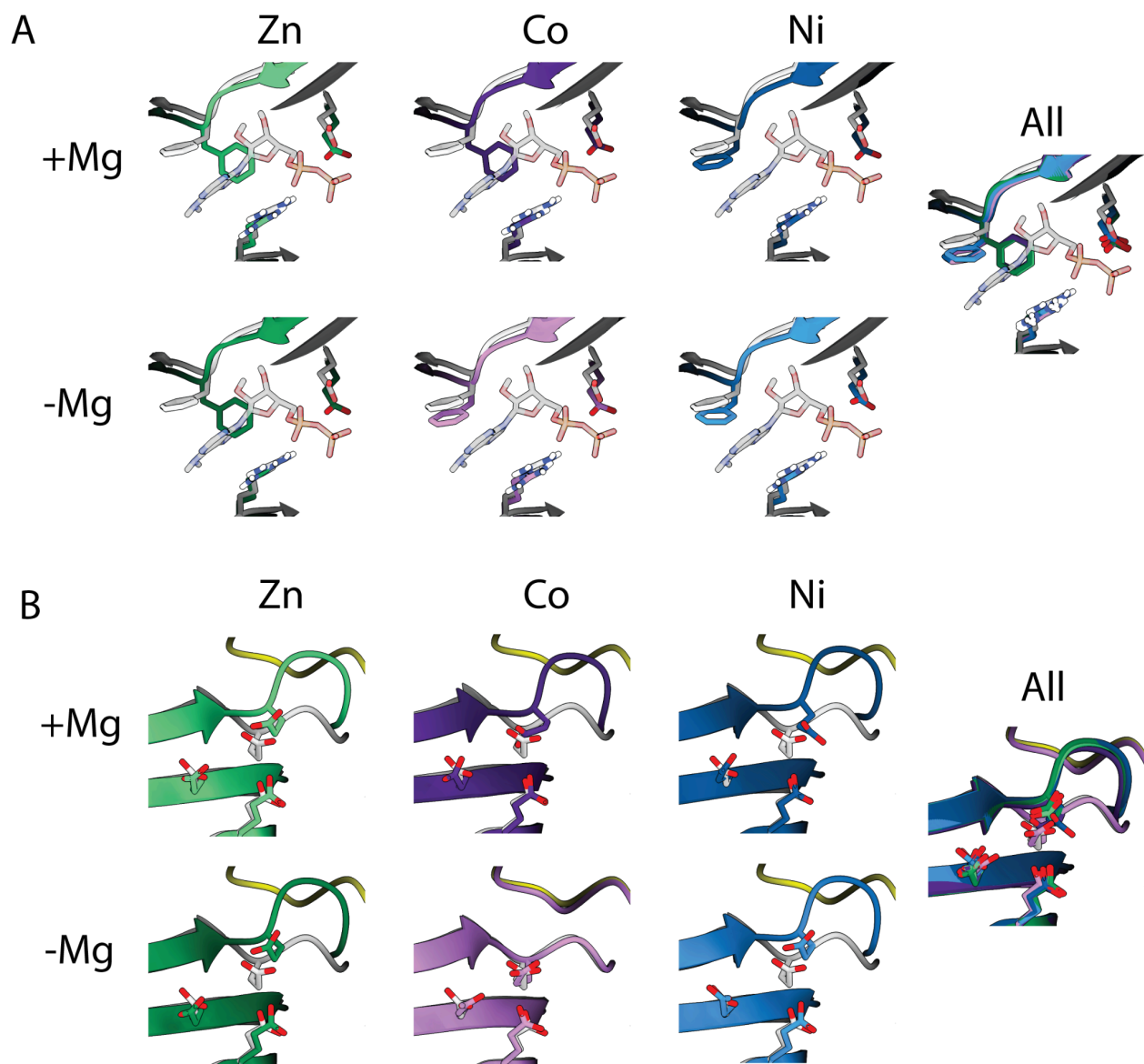


Figure 19: Nucleotide and 211 Loop Overlay.

A) Overlay of the nucleotide-binding site in Zn, Co, and Ni structures with or without Mg compared to the known structure (white) with ADP bound B) Overlay of the 211-219 loop in Zn, Co, and Ni structures with or without Mg compared to the known structure (white) with the 154-190 residues in yellow.

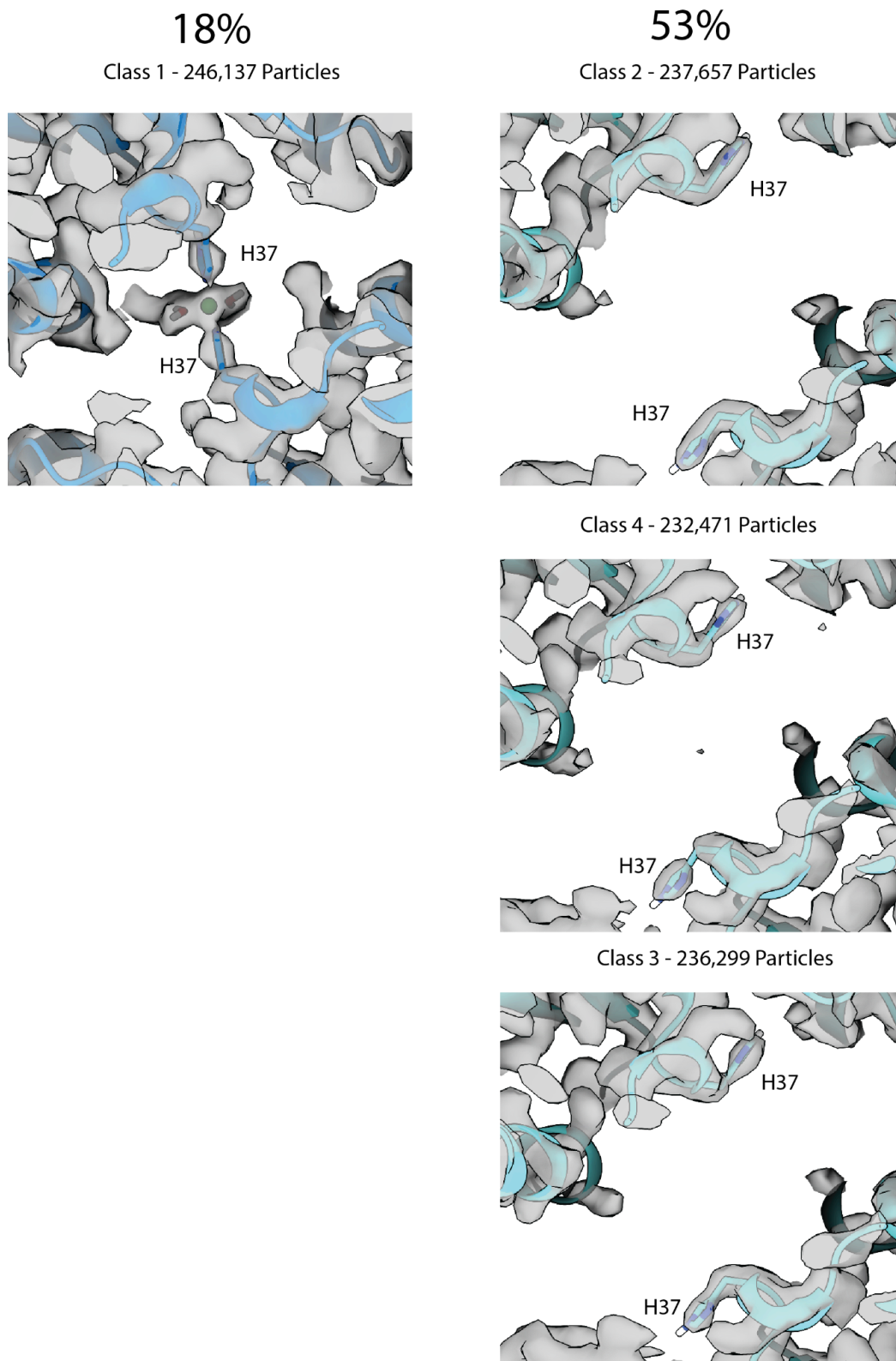


Figure 20: Ni H37 Interface Contact Ratio.

Percentage of particles with and without the H37A contact in the Ni-only filament structure after 3D classification and local refinement

Table 2: *E. coli* GS Structure Metrics

#_Name	Zn_Mg_Filament	Zn_Filament	Co_Filament	Co_Dodecamer	Ni_Filament	Ni_Dodecamer
EMDB						
PDB						
Data collection and processing						
Magnification	105000x	105000x	105000x	105000x	105000x	105000x
Voltage (kV)	300	300	300	300	300	300
Electron exposure (e-/Å ²)	39.2	39.2	50	50	50	50
Defocus range (µm)	-0.8 to -1.8 um	-0.8 to -1.8 um	-0.8 to -1.8 um	-0.8 to -1.8 um	-0.8 to -1.8 um	-0.8 to -1.8 um
Pixel size (Å)	0.843	0.843	0.4215	0.4215	0.4215	0.4215
Symmetry imposed	D6	D6	D6	D6	C1	D6
Initial particle images (no.)	1427585	3152716	1584613	1584613	1312415	1312415
Final particle images (no.)	184,453	622,256	141770	67428	246137	24808
Map resolution (Å)						
FSC threshold	2.41	2.31	2.29	2.58	2.54	2.79
Refinement						
Initial model used (PDB code)						
Model resolution (Å)	2.41	2.31	2.29	2.58	2.54	2.79
FSC threshold						
Map sharpening B factor (Å ²)	85.5	67.1	78.3	95.7	72	73.6
Model composition						
Non-hydrogen atoms						
Protein residues						
Ligands						
<i>B factors (Å²)</i>	6357; 412; Water:1 ZN:3; MG:2	6435; 416; Water:1; ZN:4	6879; 448; Water:1; CO:3		25583; 1656; Water:6; NI:11	6394; 414; NI:3
<i>Protein Ligand Water</i>	19.73/68.24/37.62; 30.81/91.41/60.11; 40.42/40.42/40.442	15.83/58.19/28.12; 50.32/63.92/57.66; 39.98/39.98/39.98	16.33/46.39/28.66; 38.37/63.99/48.39; 27.23/27.23/27.23		19.96/66.90/39.53; 49.81/85.27/59.68; 49.13/57.84/52.24	34.22/66.08/47.27; 62.94/69.81/67.14
R.m.s. deviations						
Bond lengths (Å)						
Bond angles (°)	0.003; 0.542	0.004; 0.664	0.003; 0.591		0.002; 0.492	0.002; 0.514
Validation						
MolProbity score						
Clashscore						
Poor rotamers (%)	0.95; 1.89; 0.59	0.87; 1.40; 0.0	0.95; 1.89; 0.54		0.99; 2.15; 0.66	1.02; 2.03; 0.87
Ramachandran plot						
Favored (%)						
Allowed (%)						
Disallowed (%)	98.02; 1.98; 0.0	98.28; 1.72; 0.0	98.42; 1.58; 0.0		98.28; 1.72; 0.0	97.78; 2.22; 0.0

Table 3: *E. coli* GS Structure Metrics Cont.

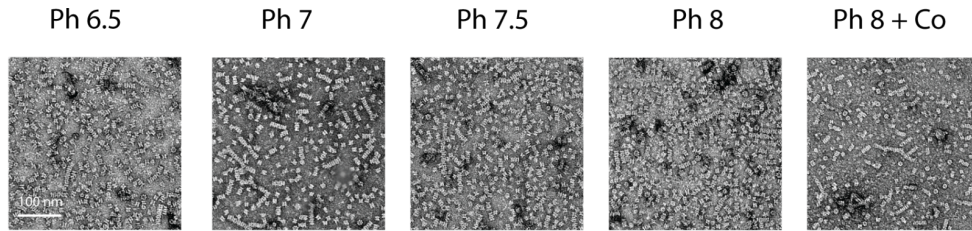
#_Name	Co_Mg_5mMATP	Ni_Mg_Filament	Co_Mg_50Glu	Co_Mg_Filament	Mg_Active_SM-Dodecamer	Co_SM-Filament	Mg_SM-Dodecamer
EMDB							
PDB							
Data collection and processing							
Magnification	45000x	105000x	105000x	105000x	105000x	105000x	105000x
Voltage (kV)	200	300	300	300	300	300	300
Electron exposure (e-/Å ²)	50	50	50	50	49.2	50	50
Defocus range (µm)	-0.8 to -1.8 um	-0.8 to -1.8 um	-0.8 to -1.8 um	-0.8 to -1.8 um	-0.8 to -1.8 um	-0.8 to -1.8 um	-0.8 to -1.8 um
Pixel size (Å)	0.4425	0.4215	0.4215	0.843	0.4215	0.4215	0.4215
Symmetry imposed	D6	D6	D6	D6	D6	D6	D6
Initial particle images (no.)	655239	3030823	1363208	1911299	2131433	1053179	2570974
Final particle images (no.)	84165	663233	112356	262186	392909	234896	143493
Map resolution (Å)							
FSC threshold	2.75	2.39	2.08	2.08	2.78	2.6	2.78
Refinement							
Initial model used (PDB code)							
Model resolution (Å)							
FSC threshold	2.75	2.39	2.08	2.08	2.78	2.6	2.78
Map sharpening B factor (Å ²)	110	98.5	54.5	65.1	102.4	105.5	93.7
Model composition							
Non-hydrogen atoms							
Protein residues							
Ligands							
B factors (Å ²)	7138; 462; MG-2; ADP-1	6372; 413; Ni-1; MG-2	6887; 449; MG-2	6373; 413; CO-2; MG-2	6901; 447; MG-1; ADP-1		
Protein Ligand Water	18; 1475; 3038; 23; 31; 23	10; 89; 45; 60; 26; 08;	12; 80; 39; 47; 25; 25;	14; 98; 47; 88; 28; 16;	28; 09; 66; 54; 45; 95; 44; 10		
R.m.s. deviations	141.27/40.70	18.97/47.18/30.28	18.40/19.58/18.99	23.47/65.85/42.06	149.93/49.72		
Bond lengths (Å)							
Bond angles (°)							
Validation	0.003; 0.571	0.002; 0.506	0.003; 0.617	0.002; 0.509	0.014; 1.474		
MolProbity score							
Clashscore							
Poor rotamers (%)	1.04; 2.24; 0.52	1.04; 2.51; 0.88	0.96; 1.89; 0.54	0.85; 0.79; 1.17	1.40; 3.48; 0.0		
Ramachandran plot							
Favored (%)							
Allowed (%)							
Disallowed (%)	97.82; 2.18; 0.0	98.27; 1.73; 0.0	98.42; 1.58; 0.0	97.78; 2.22; 0.0	96.15; 3.17; 0.68		

Chapter 3: Human Glutamine Synthetase Filaments

A) APO Human GS can Form Filaments

The human GS gene was ordered as a gene fragment and purified using a 6xHIS-SUMO tag followed by NI-IMAC and SEC. Negative stain imaging was used to screen the purified protein for proper assembly into the functional decameric oligomer (**Figure 21A**). Interestingly, during initial screening human GS was found to assemble into filaments in buffer conditions alone (50 mM Tris pH 8, 150 mM NaCl, 10 mM MgCl, 5 mM BME) (**Figure 21A**). Under negative stain the decamers appear to stack head to head, similar to the *E. coli* dodecameric oligomers (**Figure 5A**). However, complete assembly into filaments was not observed, a large portion remained as free decamers. Additionally, the filaments were relatively short compared to those observed in *E. coli* (**Figure 5A, 21A**). As an attempt to promote complete assembly, the protein was imaged under various buffer conditions. Adjusting the Ph from 6.5-8 in 0.5 step increments did not affect assembly under negative stain (**Figure 21A**). The addition of 10mM of Co also had no effect, showing that assembly was not divalent cation dependent, as was seen with *E. coli* GS. Substrates were then tested to observe their effects on filament assembly. Again, substrates appeared to have no appreciable effect on filament assembly (**Figure 21B**) compared to plain buffer conditions.

A



B

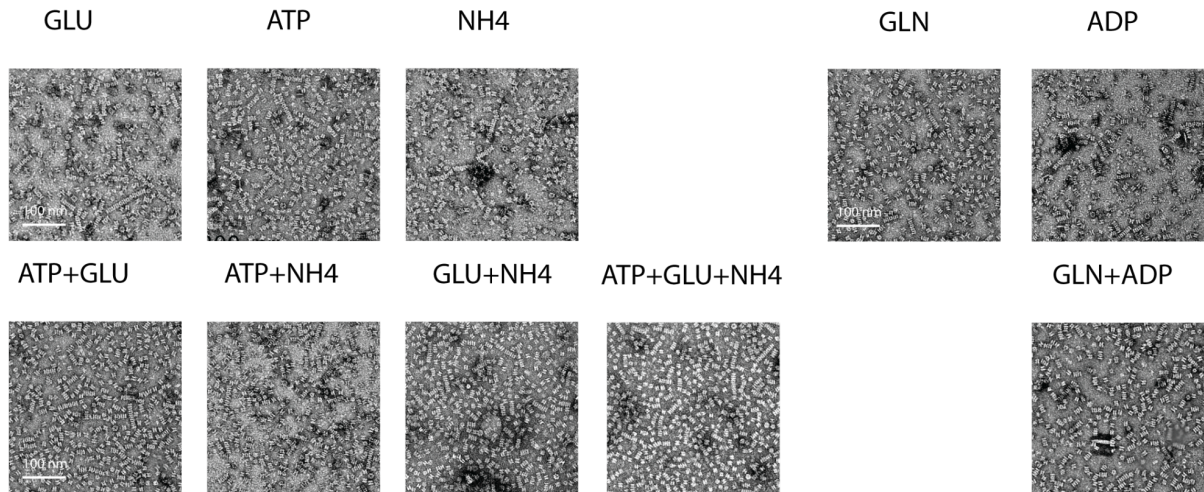


Figure 21: APO human GS Filament Conditions.

A) Negative Stain of human GS at various pH's and 1 mM Co supplementation B) Negative Stain of Human GS with substrates (left) and products (right).

B) K52 and C53 Coordinate Filaments

To identify the APO human GS interface residues cryo-EM was used to resolve a structure of the filament form of human GS to a resolution of 2.23 Å (**Figure 22A**). As predicted from the negative stain images, the filament was composed of decamers stacked head to head with one another (**Figure 22A**). The increased resolution with cryo-em enabled showed the interacting decamers were stacked with a relative rotation of 26 degrees to one another. Additionally, the only notable contact in the structure was a C5 symmetric interaction between a loop protruding from the top of each monomer consisting of residues 52-Lys-Cys-Val-Glu55 (**Figure 22B**). This loop contains residue K52, which is known to facilitate the association between decameric human GS and the BEST2 ion channel (Owji et al, 2022). K52 does not appear to be making a direct interaction with another residue. The structure instead shows C53 and C53' near one another (**Figure 22B**). This opens the possibility of the filament being held together by disulfide bonds. Additionally, this suggests the 52-55 loop may be facilitating numerous associations between human GS and other proteins. To see the influence of K52 and C53 on filament formation K52 was mutated to an alanine and C53 was mutated to an alanine and serine. Negative stain imaging showed these mutations completely disrupted filament formation (**Figure 22C**).

The APO cryo-EM micrographs showed human GS as free decamers and as filaments. These species were separated during processing and a 2.38 Å structure of the decameric APO human GS was also resolved (**Figure 22D, 24**). The two structures were practically identical with an RMSD of 0.1 Å. (**Figure 22E**) Residues 52-55 were also in the same orientation and position, indicating the human APO GS decamer does not need to undergo any major changes at the interface for filaments to form (**Figure 22F**).

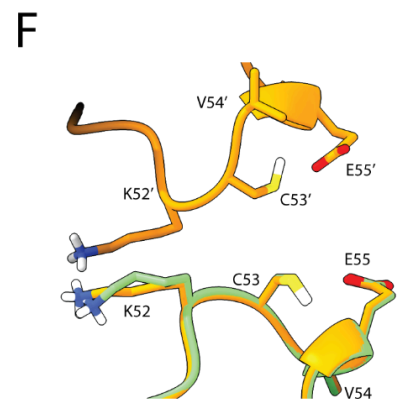
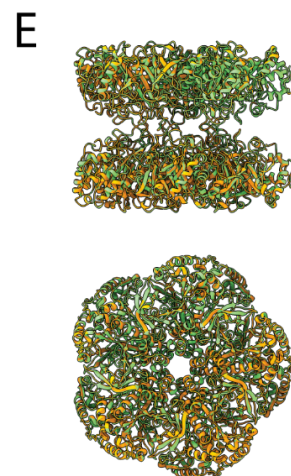
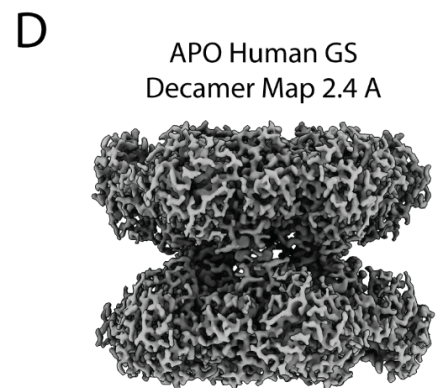
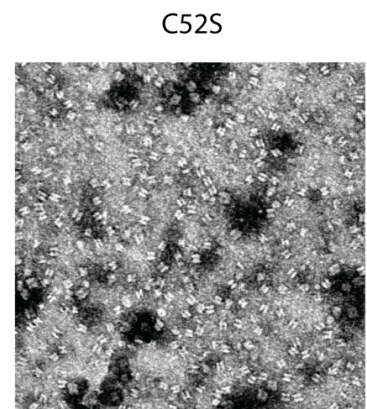
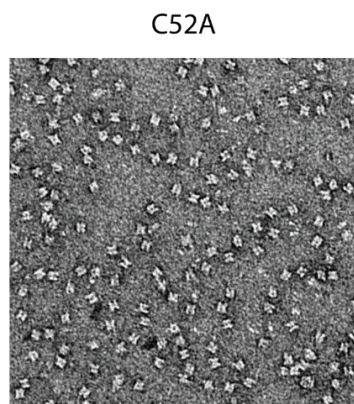
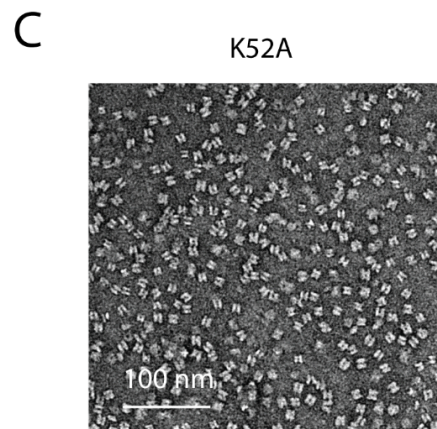
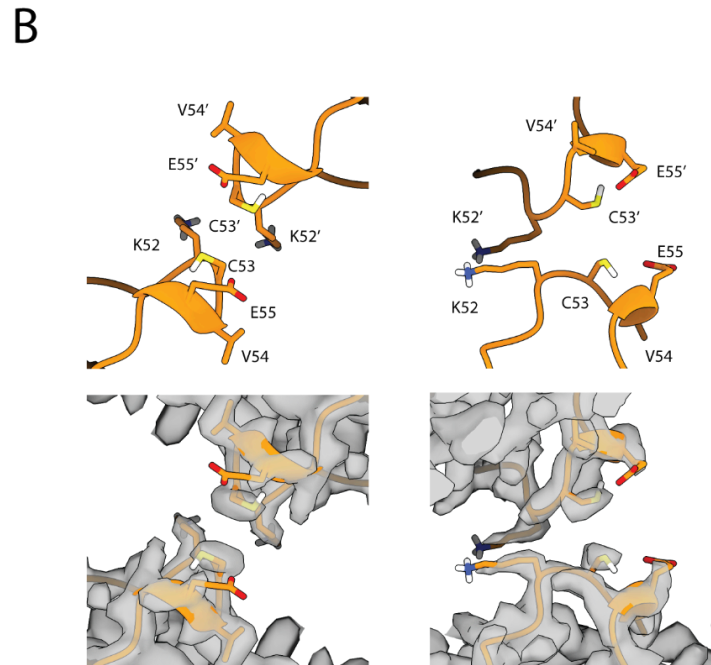
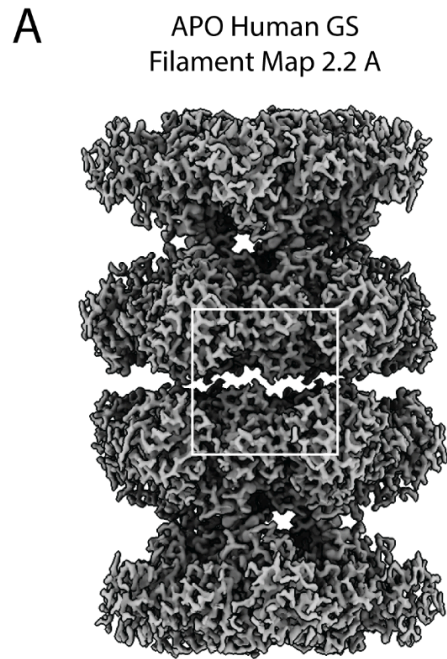


Figure 22: C53 and K52 are Required for Filament Formation.

A) Map of APO human GS with a window emphasizing the decamer interface. B) Views of the C53 loop interface with and without the APO map overlay. C) Negative stain of human GS with the K52A, C53A, and C53S mutations. D) The free decamer map of APO human GS. E) The model of the APO human filament decamer (orange) overlaid with the APO human free decamer (spindrif). F) Comparison of the C53 loops of the APO filament (orange) and free decamer (spindrif).

C) Substrates Bind to Human GS Filaments

At the resolution of negative stain, there were no identifiable structural differences between filaments in the various ligand conditions tested (**Figure 21B**). Additionally, while the active site remains intact in the APO filament, the capacity for ligands to bind human GS filaments was unknown. To evaluate binding and the effects of ligand or catalysis on filaments we collected cryo-EM samples of human GS with 5 mM ATP, turnover conditions (5 mM ATP, 10 mM Mg, 15 mM NH₄, and 50 mM glutamate), and turnover conditions with AMP-PNP instead of ATP. The concentration of substrates was selected to be 10x above the k_m values (Meek et al., 1980). ATP was the lone substrate selected because it is the first substrate to bind the active site at the top of the GS bi-funnel during catalysis (Eisenburg 2000). The ATP structure resolved to 2.1 Å, the turnover structure resolved to 2.5 Å, and the AMP-PNP structure resolved to 2.2 Å. All three structures showed nucleotides bound to human GS filaments. The ATP structure showed ATP in the active site with two Mg ions coordinated by known interacting residues E136, E203, H253, Y336, E338, and R340 (**Figure 23A**). The active turnover structure revealed ADP bound alongside two Mg ions (**Figure 23B**). The presence of ADP, and not ATP, in the turnover condition, suggests the human GS filament might hydrolyze ATP and catalyze the biosynthetic reaction. Unfortunately, no density for glutamate, the y-phosphate intermediate, or glutamine was found in the amino acid binding pocket of the turnover structure (**Figure 23D, E, F**). The AMP-PNP showed AMP-PNP occupying the ATP binding site. Similar to the productive turnover condition, glutamate was not observed in the active site (**Figure 23 C, D, E, F**). However, a third Mg ion coordinated by E136 and E203 was captured. This is the n1 site and is the metal responsible for binding glutamate. The structure of MetSox, a known inhibitor of glutamine synthetase used to mimic amino acid and intermediate binding, was used to show the location of glutamate and the y-phosphate intermediate (**Figure 23E**). No density was found to occupy this area of the three maps (**Figure 23F**).

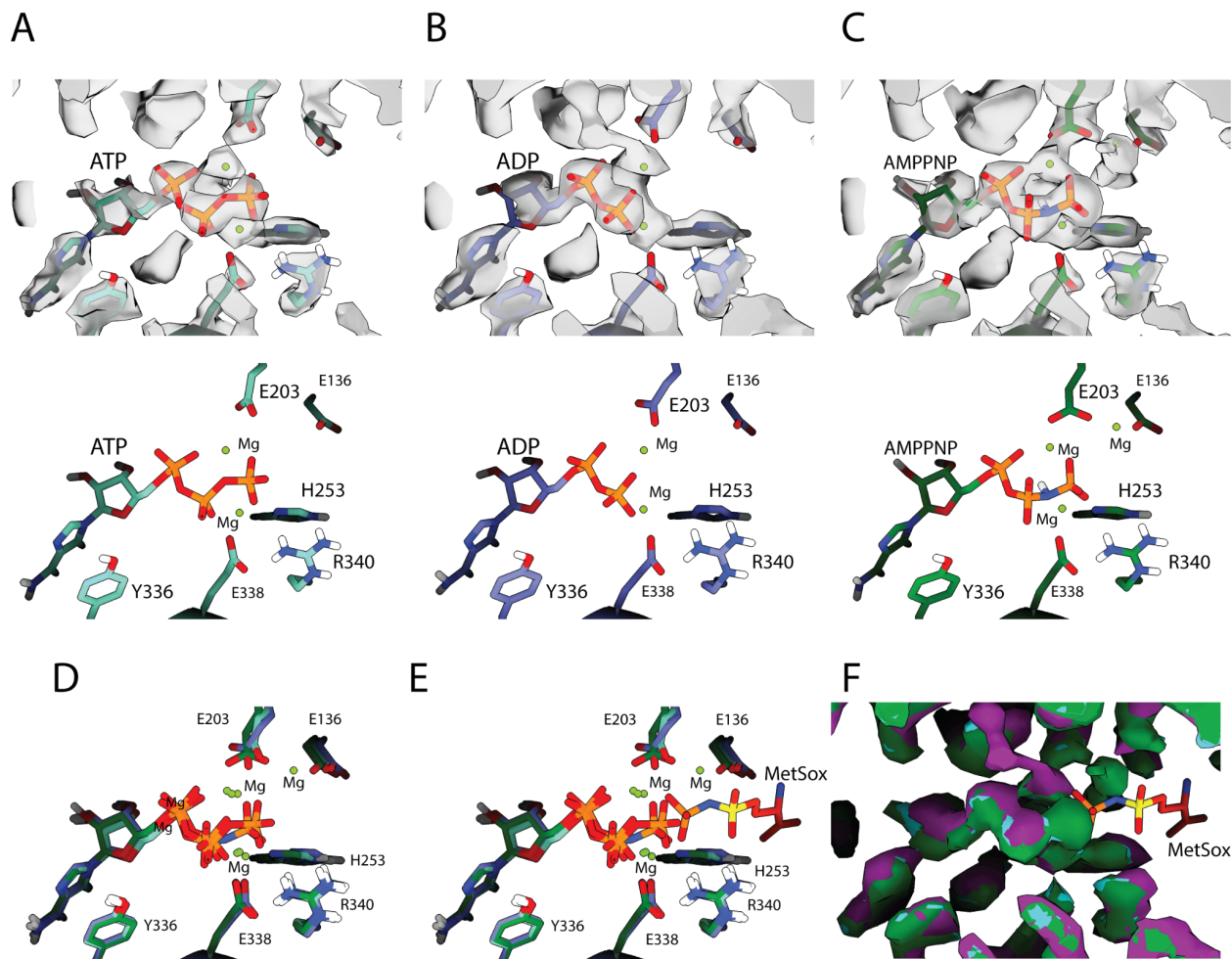


Figure 23: ATP and Turnover Active Sites.

A) Human GS ATP model (cyan, bottom) and model with map (top) with ATP bound in the nucleotide pocket. B) Human GS turnover model (plum, bottom) and model with map (top) with ADP bound in the nucleotide pocket. C) Human GS turnover AMPPNP model (clover, bottom) and model with map (top) with AMP-PNP bound in the nucleotide pocket. D) Overlay of Human GS ATP, turnover, and turnover AMPPNP models. E) D with Metsox placed in the active site. F) Overlay of Human GS ATP (cyan), turnover (plum), and turnover AMPPNP (clover) maps.

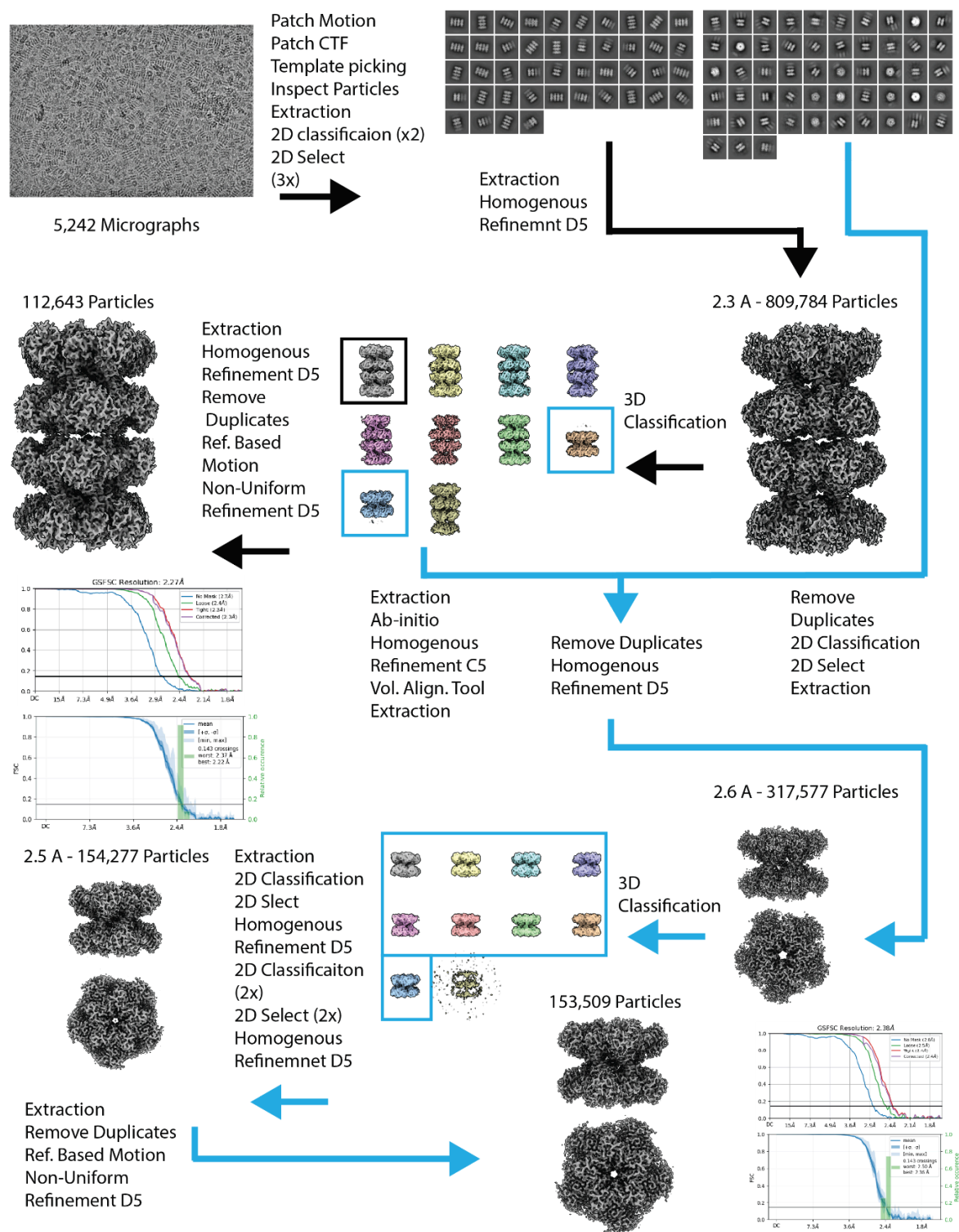


Figure 24: APO Human GS Processing.

Data processing workflow with an example micrograph, Gold Standard FSC and Conical FSC from CryoSPARC. Blue arrows indicate free decamer processing.

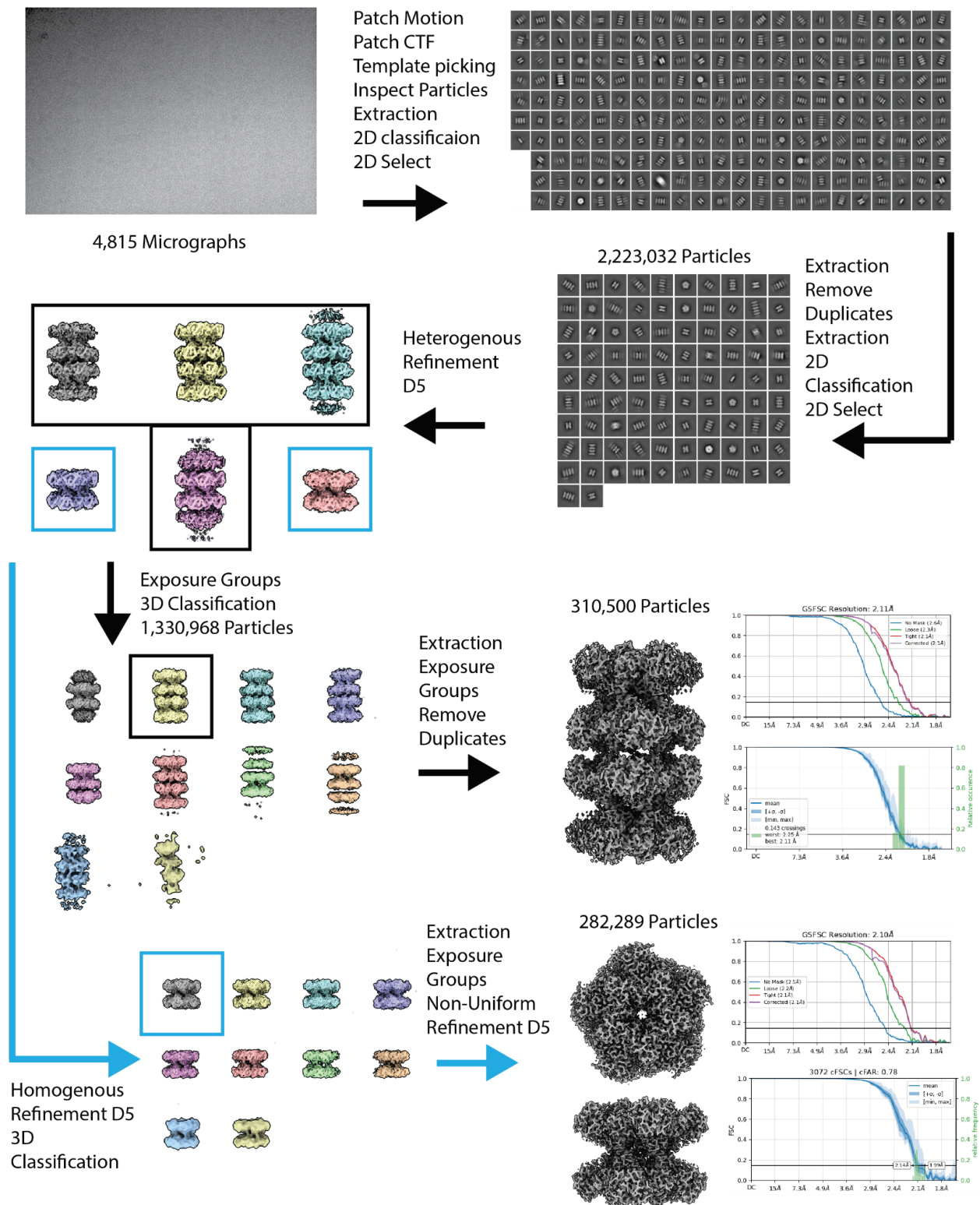


Figure 25: Human GS + ATP Processing.

Data processing workflow with an example micrograph, Gold Standard FSC and Conical FSC from CryoSPARC. Blue arrows indicate free decamer processing.

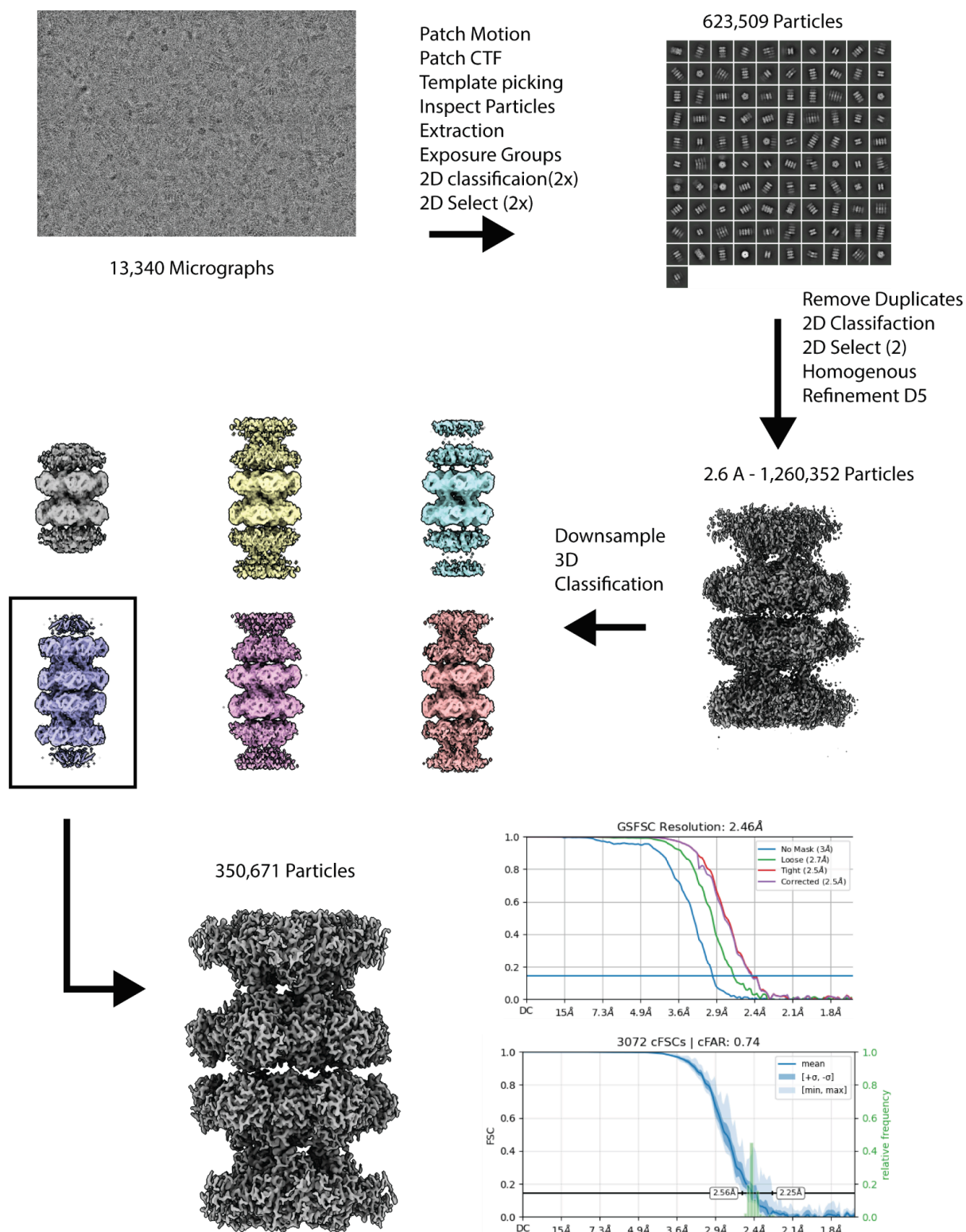


Figure 26: Human GS Turnover Condition Processing.

Data processing workflow with an example micrograph, Gold Standard FSC and Conical FSC from CryoSPARC.

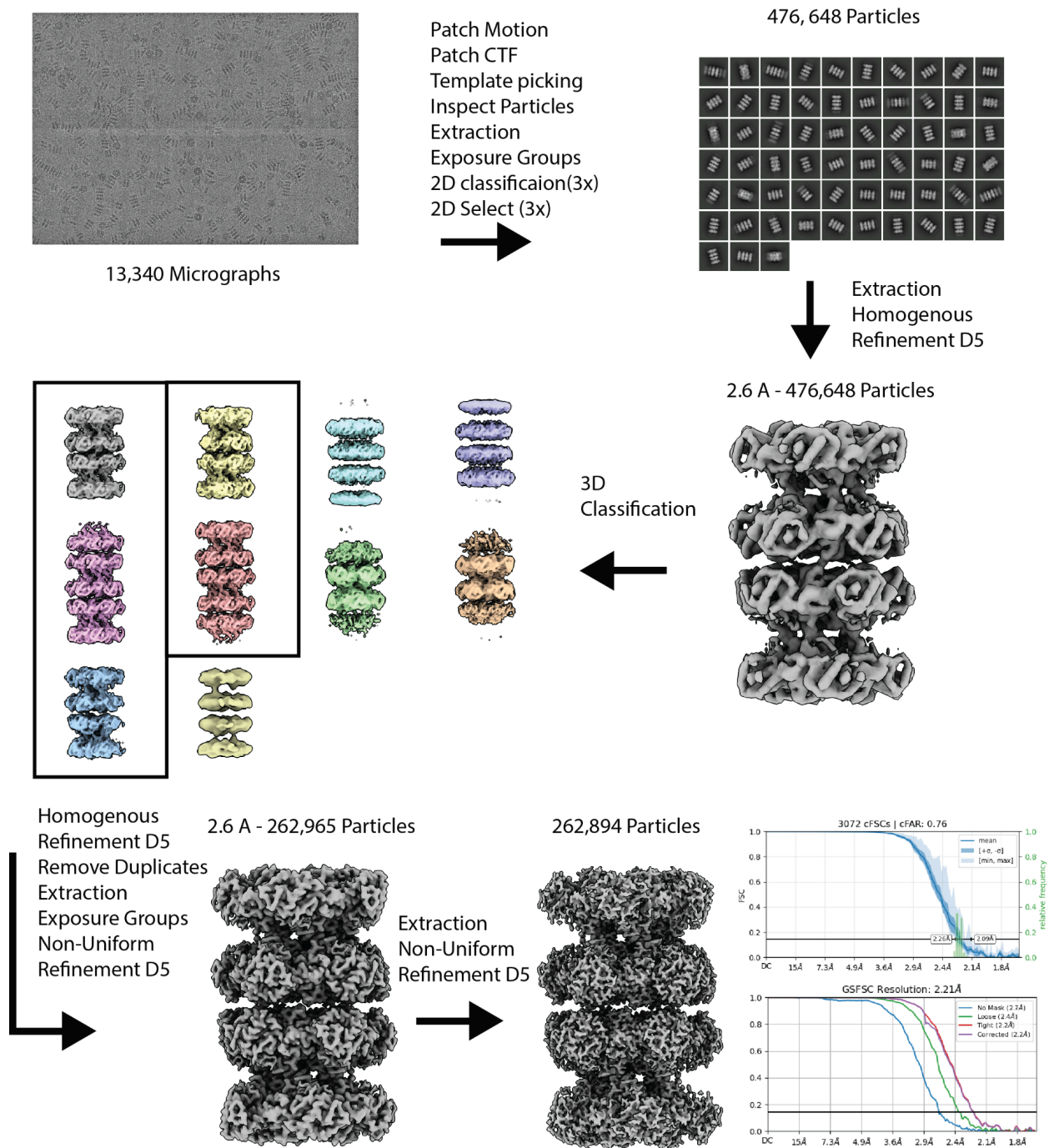


Figure 26: Human GS Turnover AMPPNP Condition Processing.

Data processing workflow with an example micrograph, Gold Standard FSC and Conical FSC from CryoSPARC.

Table 2: Human GS Structure Metrics

#_ Name	Apo Filament	Apo Decamer	ATP filament	Active Filament	AMPPNP Filament
Data collection and processing					
Magnification	105000x	105000x	105000x	105000x	105000x
Voltage (kV)	300	300	300	300	300
Electron exposure (e-/Å ²)	60	60	64.2	48.9	50
Defocus range (µm)	-0.8 to -1.8 µm	-0.8 to -1.8 µm	-0.8 to -1.8 µm	-0.8 to -1.8 µm	-0.8 to -1.8 µm
Pixel size (Å)	0.4215	0.4215	0.4215	0.4215	0.4215
Symmetry imposed	D5	D5	D5	D5	D5
Initial particle images (no.)	2591797	2591797	5923242	4669833	5667843
Final particle images (no.)	112643	153509	310500	1113494	262894
Map resolution (Å) FSC threshold	2.27 ; 0.143	2.38; 0.143	2.11; 0.143	2.26; 0.143	2.21; 0.143
Refinement					
Initial model used (PDB code)	2OJW				
Model resolution (Å) FSC threshold	2.27; 0.143	2.38; 0.143	2.11; 0.143		
Map sharpening B factor (Å ²)	77.8	95	70.2		
Model composition Non-hydrogen atoms Protein residues Ligands	114600; 7400; Water: 0; MG 20	57300; 3700; Water: 0; MG 10	54110; 3450; Water: 0; MG: 20 ATP:10		
<i>B factors (Å²)</i> <i>Protein</i> <i>Ligand</i>					
R.m.s. deviations Bond lengths (Å) Bond angles (°)	0.003; 0.457	0.004; 0.527	0.004; 0.662		
Validation MolProbity score Clashscore Poor rotamers (%)	0.97; 1.74; 0.14	1.15; 3.56; 0.26	0.87; 1.37; 0.48		
Ramachandran plot Favored (%) Allowed (%) Disallowed (%)	98.26 ; 1.74 ; 0.00	98.59 ; 1.41 ; 0.00	98.02; 1.98; 0.00		

Chapter 4: Discussion and Future Directions

The proper regulation of glutamine-glutamate cycling is important for cellular homeostasis. As the sole enzyme capable of ligating ammonium and glutamate into glutamine, GS is central to maintaining the glutamine-glutamate balance within metabolism (L. Cooper et al., 2012). Filament formation of GS has been seen in numerous species including *E. coli*, *S. cerevisiae*, *P. aeruginosa*, and *M. mazei*; spanning eukaryotes, prokaryotes, and archaea (Chek et al, 2021, Data, P., 2022; He et al., 2009; Herdering et al., 2024; Huang et al., 2022; Petrovska et al, 2014). Our work has expanded this list to include *H. sapiens*. We also show that the mechanism of filament formation in humans is unique compared to that of *E. coli*.

E. coli was long known to form filaments in the presence of metals (Valentine et al., 1968). Additionally, various metal ions influence the activity and regulation of *E. coli* GS (Abell et al., 1991). We sought to determine the mechanism of filament formation in *E. coli* GS, uncovering that Zn, Ni, and Co, coordinate filament assembly slightly differently. All three metals utilize the N-terminal H1 helix, previously identified by Huang et al. (2022), for filament formation through interaction with histidine residues 5 and 13. However, we show that metal cations play two roles, first, they coordinate filament assembly, and second, they lead to the loss of active site integrity.

Through cryo-EM, we learned that the H1 helix can accommodate several cation binding positions depending on the metal ions present (**Figure 9E**). In the case of Co, one divalent cation is bound to each histidine-5 residue at the H1 interface (**Figure 5D**), while Zn is bound to the histidine-13 residue (**Figure 9C**). The flexibility of the H1 interface is supported by the lack of signal in the Ni-induced filament map (**Figure 9C**). This suggests the Ni H1 interface is very heterogeneous compared to the rest of the filament structure which is well resolved at all residues except those known to be dynamic and involved in turnover (Armache et al., 2019, Eisenberg et al., 2000). The Ni H1 interface residues and metals may sample multiple states that all stabilize robust filament formation. Support for interface heterogeneity was the identification of the novel H37-H37 interaction (**Figure 7D**). We classified this new contact as a minor interface interaction because it was only present in 18% of interface contacts along the stacked dodecamers and only occurred under Ni conditions (**Figure 9F, 20**). Additionally, the H37A mutant did not appear to alter the capacity of filament assembly under negative stain, while the H5A and H13A mutants greatly disrupted filament assembly under all conditions (**Figure 12A**). However, the H5A and H13A mutants still formed short filaments under Zn (**Figure 12A**). This means that, like Ni, Zn may also be able to sample multiple H1 interface configurations, and filament assembly under Zn is more resistant to H1 residue mutations.

Next, we show that under the physiologically relevant Mg stabilized dodecamer, Ni, Zn, and Co supplementation causes changes to active site secondary structures (Abell et al., 1991, Eisenberg et al., 2000) (**Figure 19B**). All three metal cations bind near the active site to histidine 212 from one protomer and histidine 31 from the neighboring protomer, which in

conjunction with the first protomer forms the active site (**Figure 10E, G**). This binding causes a shift in residues 211-219 that causes this loop to occupy the region intended for residues 154-190. Residues 154-190 are unresolved, suggesting improper folding. The loss of these residues leads to the loss of the ammonium binding site. Also, the 211-219 loop shift alters the glutamate binding site. The 211-219 loop shift repositions E213 away from E221 and E132, which normally together bind the metal ion that correctly positions glutamate into the active site (**Figure 10K, L, 19B**). This active site metal is seen bound instead between E213 and H211 potentially leading to improper positioning or loss of glutamate binding in the active site. The changes to the binding site of ammonium and glutamate support the loss of activity of the enzyme. This puts forth a model that filament formation of *E. coli* GS facilitated by Ni, Zn, and Co leads to protein inactivation.

Further work needs to be done to probe the exact enzymatic consequences of metal-dependent filament assembly of *E. coli* GS. The first is to clarify the metals perform two independent roles: filament assembly and active site alteration. This can be done by purifying the H212A mutant. We hypothesize this mutant would preserve the major H1 interface and minor H37 interface while eliminating the 211 loop shift by preventing metal binding between H212 and H31' (**Figure 10E, G**). Collecting a cryo-EM sample of this mutant under Ni, Zn, or Co and showing loops 211-219 and 154-190 maintain their native positions, while in the filament, would successfully show filament assembly is independent of the metal active site alternation. Alternatively, if the loops are still disordered, it would signify other structural changes induced by filament formation yet to be identified are leading to the 211 loop movement. This result would also indicate that H212-H31' metal binding occurs only as a result of this loop shift.

The next would be to perform enzymatic assays to demonstrate filaments have no activity, as we hypothesize based on the observed active site changes. This may be difficult because we have shown that substrates lead to the collapse of filaments. However, there are several ways to address this problem. One way is to titrate substrates so that a mixture of assembled and unassembled GS is present in the solution. By measuring activity under these conditions, and under non-assembly conditions, one could equate the difference in activity to the effect imparted by the filament form of the protein on the enzymes catalytic activity. Measuring the distribution of protein assembly could be done using mass-photometry a fast label-free technique for measuring mass and protein interaction (Graciani and Yoo, 2023). Additionally, if filament collapse takes time, one could track the lag in activity compared to the unassembled protein. Only after the complete collapse of filaments would the catalytic rate of the enzyme be expected to equal the initially unassembled GS. *In vivo* assays may also be beneficial as the concentration of divalent cations used to probe filament assembly here is greater than that expected in *E. coli*, except Zn, which can reach μM levels of free ions concentration in the cytoplasm (Outten, 2001). Using gene replacement (Madyagol et al., 2011), the effects of filament-breaking mutants, like H5A and H13A, on cell proliferation could serve as a proxy for the importance of filament formation on cellular metabolism.

Human GS showed markedly different properties to that of *E. coli* GS. Human GS was found to require no other cellular factors to form filaments (**Figure 21A**). Additionally, filament formation was preserved under saturating ligand conditions (**Figure 21B**) and the product ADP bound in the active site under reaction conditions shows filaments can undergo substrate turnover and may be active (**Figure 23B**). In partnership with our group, Greene et al. (2024) found the product of human GS, glutamine, stabilized filaments, and altered the catalytic specificity of ammonia. Greene et al. conclude that the filaments serve to negatively regulate human GS. Currently, work is being done to investigate the effects of human GS filament-breaking mutants on cell growth. Future work includes recapitulating filament formation in vivo and investigating the localization of these filaments using cryo-electron tomography (Cryo-ET). Cryo-ET has recently been used to resolve novel protein interactions in mammalian cells (Chen et al., 2023). It could be a strong tool to unearth any larger role of human GS filaments in the cell outside of regulating the GS biosynthetic reaction. With already known moonlighting roles, independent of glutamine production, dependent on heterogeneous protein interactions (Eelen et al. 2018; Owji et al, 2022; Zhao et al. 2022), human GS filaments would be an interesting candidate to visualize within the cell.

We, along with others, have shown that the three GS filaments studied from yeast (Petrovska et al., 2014), humans (Greene et al., 2024), and *E. coli* (Huang et al., 2022) all serve to, or are anticipated to, down-regulate the enzyme. The extent of down-regulation varies from tuning of catalytic output to complete disruption of the active site. The conservation of this down-regulation across multiple GS subtypes and species suggests filaments are a conserved mechanism of regulation for this family of enzymes. However, it is unknown if filament formation has persisted with GS across evolutionary time or if each species has independently evolved to form filaments. The diverse quaternary structure of GS and the low conservation of the H1 helix in prokaryotes and K52 in mammals suggest (Huang et al., 2022, Krajewski et al., 2008, Moreira et al., 2019) filament formation developed independently. Work will need to be done to solidify this hypothesis. Ancestral reconstruction of old GS proteins could be an interesting avenue to investigate the occurrence of filament formation at branching points of evolution (Siddiq et al., 2017). It could also shed light on when the moonlighting functions of GS were acquired.

Materials Methods

Molecular Cloning

The coding sequence for *E. coli* and human GS (1-469) from Integrated DNA Technologies (IDT) as a gene fragment flanked with BamHI on the N-terminus and HindIII on the C-terminus. The fragment was then amplified using PCR. The amplicon was then inserted into a Kanamycin-resistant pSMT3 bacterial expression vector using restriction digestion and ligation at the BamHI/HindIII site. The product was then transformed into TOP10 *E. coli* cells. Cells were cultured overnight on LB agar plates containing 50 µg/mL kanamycin. Individual colonies were selected and grown overnight in LB medium with 50 µg/mL kanamycin. Recombinant plasmids were purified using GeneJET Plasmid Miniprep Kit (Thermo Fisher #K0503) and confirmed using Sanger sequencing. The *E. coli* Ser-GS and human GS construct were used as the parent construct for all further vector generation. Scarless and mutant GS constructs were created using Gibson Assembly or Q5 Site-Directed Mutagenesis Kit (NEB #E0554S) followed by vector amplification and sequence confirmation as stated above. Primers for Q5 mutagenesis were designed using NEBaseChanger.

Expression and Purification

WT and mutant *E. coli* and human GS with an N-terminal 6xHisSumo tag were cloned into a pSMT3-Kan vector and transformed into BL21 (DE3) RIL cells. Cells were grown in Luria broth at 37°C to an OD₆₀₀ between 0.6-0.8, then induced with 1mM IPTG and transferred to 18°C overnight. Cells were pelleted and stored at -80°C. For purification, pellets were resuspended in lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 20 mM Imidazole, 10% v/v glycerol, 5 mM Mercaptoethanol, supplemented with PMSF) and lysed using an Avisten Emulsiflex-05 homogenizer. The soluble fraction was separated using centrifugation and purified using nickel affinity chromatography (Thermo Scientific HisPur Ni-NTA Resin). Resin was washed with 50 mL of lysis buffer and eluted in 5 mL fractions with elution buffer (50 mM Tris pH 8, 150 mM NaCl, 200 mM Imidazole, 10% v/v glycerol, 5 mM Mercaptoethanol). Fractions were screened on a denaturing gel, concentrated (Millipore Amicon Ultra 30K MWCO), resuspended in imidazole-free gel filtration buffer (50 mM Tris pH 8, 150 mM NaCl, 10% v/v glycerol, 5 mM Mercaptoethanol) and incubated overnight at 4°C with ULP1. The sample was concentrated (Millipore Amicon Ultra 30K MWCO) to 500 µL and further purified by size-exclusion chromatography on an ÄKTA Pure chromatography system using a Superose 6 column (Cytiva) pre-equilibrated in gel filtration buffer (50 mM Tris pH 8, 150 mM NaCl, 10% v/v glycerol, 5 mM Mercaptoethanol). Peak fractions were concentrated (Millipore Amicon Ultra 30K MWCO) then flash frozen in liquid nitrogen and stored at -80 °C. Human gel filtration buffer contained 10 mM MgCl₂.

Negative Stain

Frozen protein samples in gel-filtration buffer were thawed at room temperature and diluted to 1 uM concentration in 50 uL assembly buffer (*E. coli* - 50 mM Tris pH 8, 150 mM NaCl; Human - 50 mM Hepes pH 7, 150 mM NaCl) incubated at room temperature before being applied to glow-discharged continuous carbon EM grids (Protochip) for 30 seconds. Grids were then washed and blotted with ddH₂O three times and stained with 0.7% uranyl formate for 30 seconds. Samples were imaged using a FEI Morgagni operating at 100 kV and a Gatan Orius CCD camera or using a FEI Tecnai G2 Spirit operating at 120 kV and a Gatan Ultrascan 4000 CCD camera with the software package Digital Micrograph (Gatan).

Cryo-EM Preparation and Data Collection

Protein samples (5-8 uM) with buffer (*E. coli* - 50 mM Tris pH 8, 150 mM NaCl; human - 50 mM Hepes pH 7, 150 mM NaCl), di-valents, and ligands as described in the results were applied to glow-discharged, Quantifoil 2/1 300 mesh gold or 2/2 400 mesh gold EM grids, blotted 1-3 times at 4 °C with 100% relative humidity, and plunge-frozen in liquid ethane using a Vitrobot vitrification system (Thermo Fisher). Data collection was performed using SerialEM on an FEI Titan Krios transmission electron microscope operating at 300 kV, with a Gatan image filter (GIF), and a K3 Summit direct electron detector.

Cryo-Em Image Processing

Movies were motion-corrected and CTF parameters were estimated in CryoSPARC Live (Punjani et al., 2017). If movies were collected in super-resolution they were binned 2X. Early template picking, using outputs from the Create Templates job and an *E. coli* dodecamer volume, 2D classification, Ab-initio, and homogenous refinement were performed in CryoSPARC Live to initially evaluate datasets. All other downstream processing was completed in CryoSPARC v4. Several rounds of 2D classification, Homogenous Refinement, Heterogenous refinement, 3D classification and Non-uniform refinement with C1, C6 or D6 symmetry were used to categorize and eliminate poorly aligned particles or noise. Recentering of particles was completed using the Volume Alignment tool and subsequent particle re-extraction. For some datasets particles were symmetry expanded and passed through another round of 3D classification, and local refinement. Processing workflows are provided for each structure. cFSC curves were produced using the Orientation Diagnostic Tool in CryoSPARC v4.

Model Building

AlphaFold model AF-POA9CS-F1 of *E. coli* glutamine synthetase was initially used for model building. Models were refined using ISOLDE in ChimeraX version 1.6.1 in conjunction with phenix.real_space_refine and phenix.validation_cryoem in Phenix. All model statistics were extracted from phenix.validation_cryoem.

Figure Assembly

All EM images were evaluated in Fiji version 2.3.0. Map and model images were prepared in ChimeraX version 1.6.1. Final figures were built in Adobe Photoshop version 25.9 and Adobe Illustrator version 28.5.

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