TECHNICAL UNIVERSITY OF MUNICH

DEPARTMENT OF CHEMISTRY

PROTEIN CHEMISTRY

Covalent capture of enzyme-substrate complexes for target identification and structure determination

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Vollständiger Abdruck der von der Fakultät für Chemie der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

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Die Dissertation wurde am 07.05.2021 bei der Technischen Universität München eingereicht und durch die Fakultät für Chemie am 30.08.2021 angenommen.

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ABSTRACT

The endoplasmic reticulum is central to the production and correct folding of a vast amount of membrane, organellar, and secreted proteins. To ensure the high quality of the produced proteins, the amount of unfolded proteins is constantly monitored and the required chaperone activity is tightly regulated. One mechanism to adapt to the fluctuating burden of unfolded proteins is the reversible AMPylation of the Hsp70 chaperone BiP. AMPylation renders BiP inactive while deAMPylation restores its chaperone activity. In metazoans both modification and demodification are carried out by the Fic-enzyme FICD/HYPE. The structural basis for FICD mediated AMPylation of BiP has remained elusive due to their transient interaction. Herein, I utilize thiol-reactive cosubstrate analogs to covalently tether FICD to its substrate. The AMPylation complex was isolated for structural characterization and its atomic structure determined via crystallography. The complex structure reveals that the specificity of FICD towards the ATP state of BiP is mediated by the TPR motifs of FICD that engage with the conserved hydrophobic linker in the nucleotide-binding domain. Both biochemical investigations and cellular assays verify the mode of interaction and led to the hypothesis that Fic-enzymes generally rely on adjacent domains and motifs to foster target recognition. Importantly, the deAMPylation of BiP depends in large parts on the same interactions identified for BiP AMPylation. In addition, the results demonstrate that neither AMPylation nor deAMPylation of BiP are directly regulated by unfolded proteins. Together, a combinatorial approach of chemical biology, X-ray crystallography, biochemistry, cell biology, and molecular dynamics simulation provided the structural basis of a central regulatory mechanism that ensures homeostasis in the ER.

The chaperone BiP represents a physiological substrate of FICD while other physiological substrates that may exist are yet to be identified. Identification of AMPylation substrates, however, still poses a major challenge in today's research since the commonly used cosubstrate derived probes suffer from competition against endogenous ATP. Herein, a novel and advantageous concept of target identification is presented. Recombinantly produced Fic-enzymes that are covalently equipped with a thiol-reactive cosubstrate analog serve as a bait to capture their substrates in cellular lysates. Indeed, in pull-down experiments with FICD the physiological AMPylation substrate BiP was enriched and identified via LC-MS/MS. Furthermore, novel AMPylation targets are identified, characterized, and confirmed *in vitro*.

While the pull-down experiments relied on recombinantly produced FICD with a common affinity tag for enrichment, a different strategy was designed that would reduce the background signal in LC-MS/MS caused by highly abundant cellular proteins. Combining thiol-reactive cosubstrate analogs and immobilization of Fic-enzymes to magnetic nanoparticles would permit enrichment of AMPylation substrates by covalent linkage alternative to commonly used affinity-based

methods. Enrichment of covalently linked AMPylation substrates is anticipated to tolerate harsh washing conditions, effectively reducing the signal-to-noise ratio in mass spectrometry. Preliminary work towards this novel concept is performed by establishing methods to reliably quantify the amount and assess the activity of the immobilized enzymes.

Furthermore, a review is presented on the characterization of transient protein-protein and protein-DNA complexes. Structural characterization of macromolecular complexes is crucial for the mechanistic understanding of biological processes and advanced applications such as drug development yet poses a great challenge due to the dynamic nature of the interactions. To tackle this challenge, a plethora of concepts were developed to stabilize transient interactions by covalent crosslinking for the structural characterization of macromolecular complexes. The review gives a categorical overview and future perspectives of crosslinking approaches in structural biology and focuses on the most recently developed methods for specific stabilization of transient macromolecular complexes.

ZUSAMMENFASSUNG

Das endoplasmatische Retikulum ist von zentraler Bedeutung für die Produktion und korrekte Faltung einer großen Anzahl von membranständigen, organellaren und sekretierten Proteinen. Um eine hohe Qualität der produzierten Proteine zu gewährleisten, wird die Menge der ungefalteten Proteine fortwährend überwacht und die erforderliche Chaperonaktivität streng reguliert. Ein Mechanismus zur Anpassung an die fluktuierende Last an ungefalteten Proteinen ist die reversible AMPylierung des Hsp70-Chaperons BiP. Die AMPylierung von BiP inaktiviert das Enzym, wohingegen die deAMPylierung dessen Chaperonaktivität wiederherstellt. In Metazoen werden sowohl die Modifikation als auch die Demodifikation durch das Fic-Enzym FICD/HYPE durchgeführt. Die strukturelle Grundlage für die FICD-vermittelte AMPylierung von BiP ist aufgrund ihrer transienten Interaktion schwer zu ergründen. In dieser Arbeit werden thiol-reaktive Cosubstrat-Analoga verwendet, um FICD kovalent an sein Substrat zu binden. Der AMPylierungskomplex wurde zur strukturellen Charakterisierung isoliert und seine atomare Struktur mittels Kristallographie bestimmt. Die Komplexstruktur zeigt, dass die Spezifität von FICD gegenüber dem ATP-Zustand von BiP durch die TPR-Motive von FICD vermittelt wird, die mit dem konservierten hydrophoben Linker in der Nukleotid-Bindungsdomäne interagieren. Mittels biochemischer und zellbasierter Untersuchungen wird der Interaktionsmodus verifiziert und die Hypothese abgeleitet, dass Fic-Enzyme generell von benachbarten Domänen und Motiven abhängig sind, um ihr Substrat zu binden. Bemerkenswerterweise hängt die deAMPylierung von BiP zu großen Teilen von den gleichen Interaktionen ab, die für die AMPylierung von BiP identifiziert wurden. Darüber hinaus zeigen die Ergebnisse, dass weder die AMPylierung noch die deAMPylierung von BiP direkt durch ungefaltete Proteine reguliert werden. Schlussendlich führte ein kombinatorischer Ansatz aus chemischer Biologie, Kristallographie, Biochemie, Zellbiologie und Molekulardynamiksimulation zu strukturellen Einblicken in einen wichtigen Regulationsmechanismus, der die ER-Homöostase sicherstellt.

Das Chaperon BiP stellt ein physiologisches Substrat von FICD dar, während andere physiologische Substrate, falls existent, noch nicht identifiziert wurden. Die Identifizierung von AMPylierungs-Substraten stellt jedoch immer noch eine große Herausforderung in der heutigen Forschung dar, da die üblichen, vom Cosubstrat-abgeleiteten, Sonden unter der Konkurrenz zu endogenem ATP leiden. Hier wird ein neuartiges und vorteilhaftes Konzept der Substrat-Identifizierung vorgestellt. Rekombinant hergestellte Fic-Enzyme, die kovalent mit einem thiol-reaktiven Cosubstrat-Analogon ausgestattet sind, dienen als Köder, um ihre Substrate in zellulären Lysaten einzufangen. In der Tat wurde in Pull-down-Experimenten mit FICD dessen physiologisches AMPylierungssubstrat BiP angereichert und über LC-MS/MS identifiziert. Darüber hinaus werden neuartige AMPylierungssubstrate identifiziert, charakterisiert und *in vitro* bestätigt.

Während sich die Pull-down-Experimente auf rekombinant hergestelltes FICD mit einem gewöhnlichen Affinitäts-Tag für die Anreicherung stützten, suchten wir nach einer anderen Strategie, die das Hintergrundsignal in der Massenspektrometrie, das durch hochabundante zelluläre Proteine verursacht wird, reduzieren würde. Die Kombination von thiol-reaktiven Cosubstrat-Analoga und die Immobilisierung von Fic-Enzymen an magnetischen Nanopartikeln würde die Anreicherung von AMPylierungssubstraten durch kovalente Bindung, im Gegensatz zu den üblicherweise verwendeten affinitätsbasierten Methoden, ermöglichen. Es ist zu erwarten, dass die Anreicherung von kovalent verknüpften AMPylierungssubstraten harsche Waschbedingungen toleriert und somit das Hintergrundsignal in der Massenspektrometrie deutlich reduziert. Vorarbeiten zu diesem neuartigen Konzept werden durch die Etablierung von Methoden zur zuverlässigen Quantifizierung der Menge und Bewertung der Aktivität des immobilisierten Enzyms durchgeführt.

Weiterhin wird ein Überblicksartikel über die Charakterisierung von transienten Protein-Protein- und Protein-DNA-Komplexen präsentiert. Die strukturelle Charakterisierung von makromolekularen Komplexen ist entscheidend für das mechanistische Verständnis biologischer Prozesse und fortschrittliche Anwendungen wie die Entwicklung von Medikamenten, stellt jedoch aufgrund der dynamischen Natur der Wechselwirkungen eine große Herausforderung dar. Um dieser Herausforderung zu begegnen, wurde eine Fülle von Konzepten entwickelt, um transiente Wechselwirkungen durch kovalente Verknüpfung für die strukturelle Charakterisierung von makromolekularen Komplexen zu stabilisieren. Der Übersichtsartikel gibt einen kategorischen Überblick über Konzepte der kovalenten Verknüpfung in der Strukturbiologie und fokussiert auf die zuletzt entwickelten Methoden zur spezifischen Stabilisierung von transienten makromolekularen Komplexen.

1. INTRODUCTION

Proteins are fundamental to life since most biological processes are governed by them. Proteins serve as building blocks for macromolecular structures and as enzymes that catalyze diverse chemical reactions. The three-dimensional structure and thus the function of proteins is determined by the primary sequence of amino acids.[1] The covalent modification of amino acids, referred to as posttranslational modification (PTM), is one example of many mechanisms that account for the functional plasticity of proteins that are based on merely 20 proteinogenic amino acids.

The following sections of the introduction will provide the background information on enzymes and substrates that are involved in a particular PTM, namely AMPylation. Furthermore, the challenges and recent progress in both identification of AMPylation substrates and the structural characterization of low-affinity complexes will be presented.

1.1 Protein AMPylation

The transfer of AMP to molecules is commonly known and understood as a means to activate metabolites/proteins for subsequent reactions (e.g. synthesis of aminoacyl-tRNA for protein synthesis or ubiquitination).[2] The transfer of AMP to proteins as a PTM, however, was barely studied until 10 years ago and exhibits a regulatory function. The modification of proteins with an AMP moiety (from here on referred to as AMPylation) by an AMP-transferase is dependent on ATP as a cosubstrate that provides the AMP moiety for transfer as well as the energy to drive the reaction.[3] The AMP moiety is transferred to a hydroxyl-bearing side chain in the protein substrate, typically a threonine or tyrosine residue (**Figure 1**).[4,5] AMPylation was first described in the late 1960s when researchers discovered that the activity of the *Escherichia coli* glutamine synthetase is controlled by AMPylation and thereby adjusted to the cellular availability of nitrogen.[6–9] While the AMP-transferring enzyme glutamine synthetase adenylyl transferase (GS-AT) consists of a DNA polymerase β-like fold, the breakthrough in the field of AMPylation was achieved by the discovery of the AMPylation activity of the class of Fic-enzymes.[10] The bacterial effector protein VopS that belongs to the class of Fic-enzymes was linked to cytoskeleton collapse during infection of host cells with *Vibrio parahaemolyticus*.[10] In 2009, the researchers were able to demonstrate that Rho GTPases such as Cdc42 that regulate cytoskeleton dynamics are inactivated by covalent modification with AMP by VopS.[10] This study reopened the scientific field of AMPylation and paved the way for many more important discoveries. In the following years, AMPylation of small GTPases was established as a common way for pathogens to interfere with host signaling thus facilitating the uptake of intracellular bacteria.[11–14] Moreover, the only human representative of Fic-enzymes, namely FICD/HYPE, was identified[10,13] and recent studies shed first light on the physiological role of FICD in protein homeostasis by FICD mediated AMPylation of the ER-resident Hsp70 chaperone

BiP.[15–18] Only recently, pseudokinases (kinase-related enzymes devoid of phosphorylation activity) have been demonstrated to possess AMPylation activity.[19,20] To summarize, three different classes of enzymes are identified as AMPylases to date: enzymes of a DNA polymerase β-like fold, Fic-enzymes, and pseudokinases (**Figure 1**).[10,12,19]

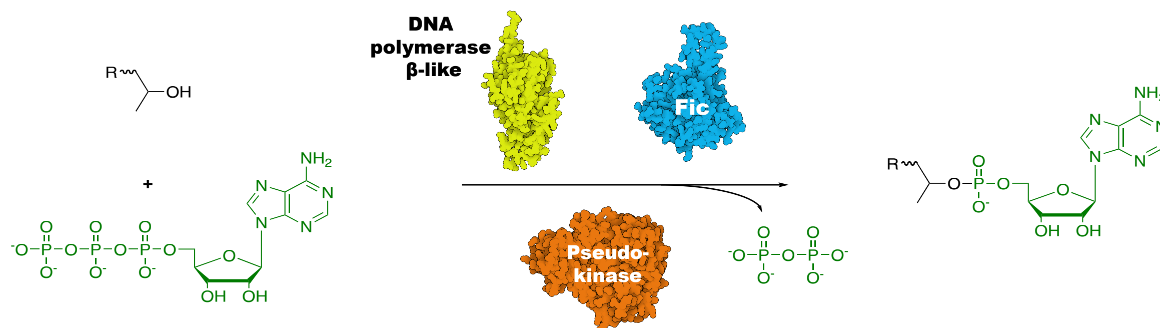

***Figure 1** | **AMPylation as posttranslational modification.** The reaction is dependent on ATP as cosubstrate and catalyzed by three different classes of enzymes. The AMP moiety is transferred to hydroxyl-bearing amino acids (Ser, Thr, Tyr) in the protein substrate R.*

1.2 Structural and functional aspects of AMPylation

1.2.1 Structure and function of Fic-enzymes

The name of Fic (filamentation induced by cAMP) enzymes originates from the observation that a mutant of the *E. coli* representative of Fic-enzymes induced a filamentous and cell division defective phenotype when the bacteria were grown at elevated temperatures.[21–23] Despite the low sequence conservation of Fic-enzymes, they are easily identified by two hallmarks. First, they share a conserved amino acid consensus sequence HxFx(D/E)GNGRxxR (referred to as Fic-motif) which is instrumental for their catalytic activity.[10,24] Second, they exhibit a remarkably conserved core structure that comprises eight α-helices that surround the central catalytic loop (**Figure 2**).[25] Of note, Fic-enzymes are also considered as members of the Fido superfamily that explicitly includes the related protein families of doc (death on curing) and AvrB which possess a noncanonical Fic-motif and an incomplete structural core.[25] In addition to the conserved structural core, Fic-enzymes often display further structural elements that are thought to be involved in substrate binding such as the β-flap within the Fic core structure or other adjacent domains.[26,27] Another structural element that is common to about 90% of all Fic-enzymes is the so-called inhibitory α-helix (α_{inh}) that autoinhibts the enzyme's catalytic activity.[28,29] Like the Fic-motif, the α_{inh} is characterized by an amino acid consensus motif (S/T)XXXE(G/N).[28,30] A remarkable study, which represented a major breakthrough in the field of AMPylation and Fic-enzymes, demonstrated that the substitution of the conserved glutamate by a glycine relieves the autoinhibition of Fic-enzymes.[28] Based on this finding, proteins of the Fido superfamily were classified into three classes according to the position of the inhibitory helix relative to the catalytic

Fic-motif (**Figure 2**).[28] Class I represents exclusively bacterial enzymes that are part of toxin/antitoxin modules in which the Fic-motif is present in the toxin whereas the α_{inh}-module is part of a separate antagonistic protein, the antitoxin. Class II represents the largest class and comprises about 80% of all Fic-enzymes. Therein, the α_{inh} is located N-terminal of the Fic-motif. In class III Fic-enzymes, the α_{inh} locates to the C-terminus.[27,28] A small number of Fic-enzymes that mostly represent toxins of pathogenic bacteria do not contain an α_{inh}-module which makes them constitutively active as part of the pathogen's frontal attack strategy towards the host.[27,31]

Since Fic-enzymes share the catalytically relevant Fic-motif (HxFx(D/E)GNGRxxR) they are considered to share the same enzymatic mechanism (**Figure 2**). With the discovery of the Fic-enzyme VopS from *Vibrio parahaemolyticus* it became apparent that the highly conserved histidine within the Fic-motif is crucial for AMP-transfer.[10] This observation was supported in many studies on diverse Fic-enzymes.[13,15,32–34] Kinetic studies on VopS mediated AMPylation and structural analysis of the complex of the Fic-enzyme IbpA from *Histophilus somni* and its target the human Rho GTPase Cdc42 elucidated the role of the Fic-motif and the catalytic histidine within:[24,35] AMP-transfer follows a sequential mechanism in which both substrate and cosubstrate (ATP) form a ternary complex rather than a ping-pong mechanism in which the ATP is covalently bound to the catalytic histidine in an intermediate state.[24,35] The important role of the catalytic histidine is based on its action as a general base to deprotonate the substrate's hydroxyl-bearing amino acid and its contribution to the correct positioning of the cosubstrate in the active site.[24,35,36] The other residues are intimately involved in interactions mainly with the triphosphate moiety of the nucleotide. The GNG sequence forms an anion hole to accommodate the charged α-phosphate during catalysis, whereas the conserved acidic side chain of glutamate/aspartate coordinates an Mg^{2+} ion to stabilize the negatively charged α –and β-phosphates.[24,28,30,37] In addition, the positively charged arginines within the Fic-motif coordinate both ribose and phosphates of the nucleotide.[24,28,35] Of note, the nucleobase of the bound nucleotide is barely involved in specific interactions with the Fic-enzyme and positioned in a hydrophobic pocket. Hence, Fic-enzymes are rather unspecific in regards to their cosubstrate which is reflected by the ability of Fic-enzymes to use different nucleotides (ATP, GTP, TTP, CTP, UTP) for nucleotide monophosphate (NMP) transfer *in vitro*.[38] Specificity for AMP-transfer is therefore mainly achieved by ATP's high cellular concentrations.[39] Remarkably, there are a few interesting exceptions regarding the cosubstrate specificity of Fic-enzymes. For instance, the Fic-protein AvrAC from the plant pathogen *Xanthomonas campestris* is very specific for its cosubstrate UTP and consequently transfers UMP instead of AMP.[40] Furthermore the Fic-enzyme AnkX from *Legionella pneumophila* specifically binds CDP-choline as a cosubstrate.[32,41] Since CDP-choline is inversely bound in the active site, phosphocholine (and not CMP) is transferred to AnkX's target Rab1b.[41] Herein, specificity for CDP-choline and its alternative binding mode are imposed by specific recognition of cytidine by an

additional CMP domain within AnkX as well as acidic residues within the nucleotide-binding pocket of the Fic domain that stabilize the positive charge of choline.[41] Of note, the catalytic mechanism of AnkX mediated phosphocholine transfer appears to differ from the sequential mechanism reported for Fic mediated NMP-transfer, as there is evidence that rather supports a ping-pong mechanism for AnkX.[42,43] Furthermore, the doc (death on curing) toxin of the toxin/antitoxin module of doc/Phd was shown to relate to phosphorylation rather than AMPylation.[44] Similar to AnkX, doc binds the cosubstrate (here ATP) in an inverted position with the γ–phosphate located in the GNG anion hole resulting in phosphate transfer instead of AMP-transfer.[45] Importantly, the binding mode is partially mediated by a degenerated Fic-motif which would cause steric clashes upon canonical cosubstrate binding.[45]

Fic-enzymes are known to exhibit only low affinity towards their dedicated protein substrates.[16,42,46] While the low affinity challenges the characterization of the interactions of Fic-enzymes with their substrates, some studies successfully identified general characteristics of protein substrate recognition. Analysis of the complex structure of IbpA with its target Cdc42 revealed that the aforementioned β–flap-module of IbpA interacts sequence independently by β-augmentation with Cdc42 and thereby directs the targeted residue to the catalytic site.[24] This observation was also supported by other studies.[36,47] Structural analysis of the Legionella effector AnkX revealed an extended β–flap that later on was found to be essential for accessing the substrate's phosphocholination site by the local unfolding of the target protein.[41,48] Another important role in the recognition of Fic-enzyme substrates is attributed to the accessory domains within Fic-enzymes (**Figure 2**).[27] IbpA, for instance, specifically interacts with Cdc42 via its arm domain.[24] A mode of interaction that is likely to apply also to IbpA's orthologue VopS due to their structural similarity.[35] The structure of AnkX covalently tethered to its substrate Rab1b and biochemical evidence illustrated the importance of the ankyrin repeats for Rab1b binding and phosphocholination.[48] Many other Fic-enzymes comprise additional domains and motifs (such as helix-turn-helix motifs, tetratricopeptide repeats, or leucine-rich repeats) that likely contribute to substrate binding, yet their role is still to be determined.[27]

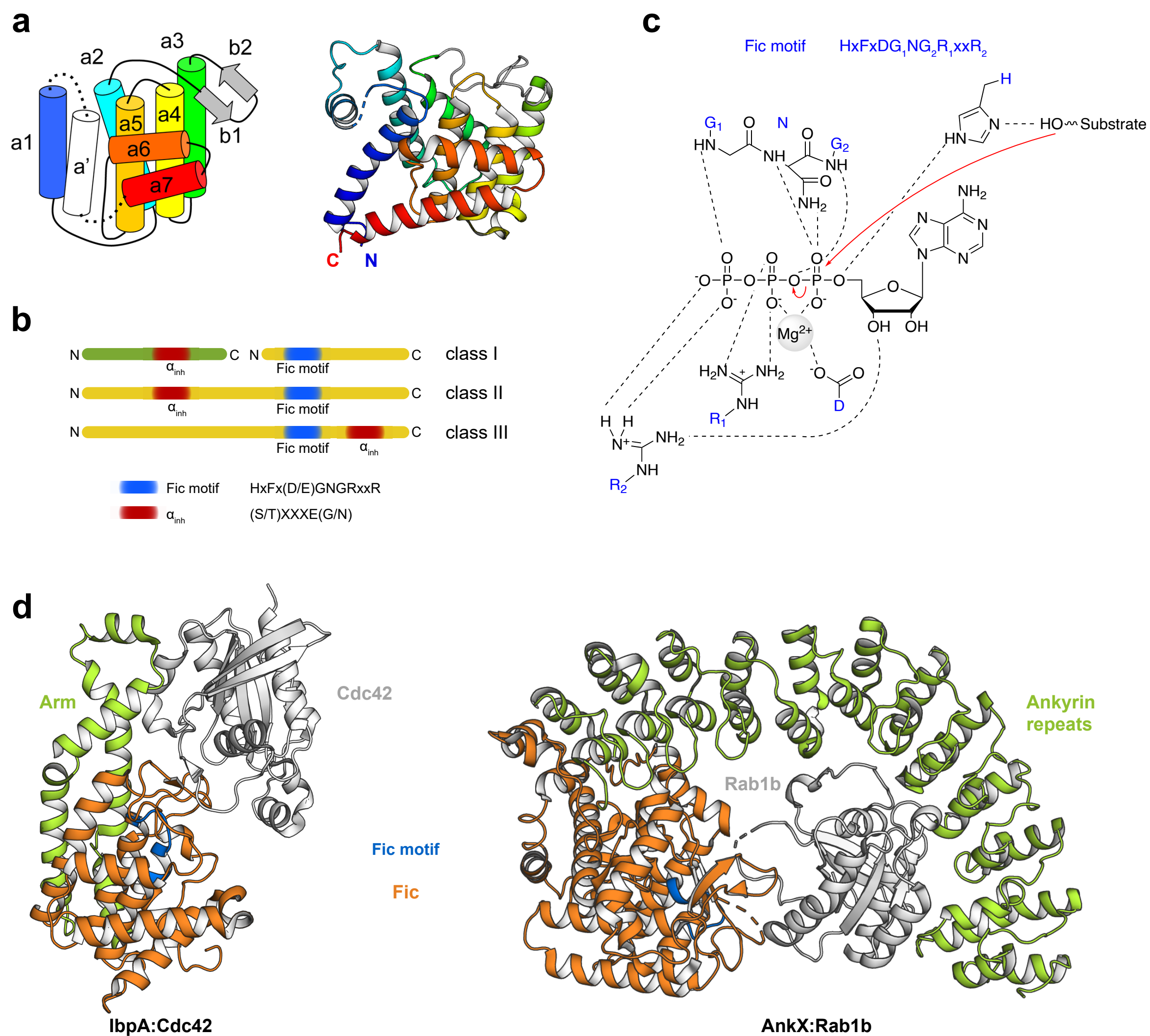

***Figure 2** | **Fic-enzymes are conserved in structure and distinct motifs.** **a)** The structural core of Fic-enzymes consists of eight α-helices. Some Fic-enzymes display an additional structural motif, the β-flap. The secondary structure elements are colored in rainbow from blue (N-terminal) to red (C-terminal). The conserved structural core is illustrated by a scheme (left panel) and FICD (right panel) (PDB 6I7J). For clarity, only the Fic domain of FICD (209-432) is displayed. The scheme on the left panel was taken from Kinch et al. 2009.[25] (CC BY 4.0) **b)** Fic-enzymes are categorized into three classes depending on the position of the α_{inh}. **c)** Schematic representation of cosubstrate (ATP) recognition and mechanism of Fic-enzymes. Adapted with permission from Hedberg & Itzen, 2014.[4] Copyright 2015 American Chemical Society. **d)** Fic-enzymes often display additional domains that are thought to involve in substrate recognition. The left panel shows the complex structure of IbpA:Cdc42 (PDB 4ITR) and the right panel the complex structure of AnkX:Rab1b (PDB 6SKU). The Fic domains are colored in orange with a blue Fic-motif. The adjacent domains are colored in green, the substrates in white.*

1.2.2 Regulation of AMPylation

As mentioned above, most Fic-enzymes are autoinhibited by the presence of the α_{inh}-module bearing the (S/T)XXXE(G/N) motif.[28,30] This layer of regulation can occur intermolecularly as exemplified by class I Fic-enzymes (toxin/antitoxin systems) or intramolecularly in regards to class II and class III Fic-enzymes. The molecular basis for autoinhibition was reported in a pioneering study in 2012.[28] The researchers identified the Fic-enzyme VbhT as part of a Bartonella toxin/antitoxin (VbhT/VbhA) system and upon structural analysis of the VbhT:VbhA complex identified a helix within VbhA that would interfere with ATP binding in VbhT. Strikingly, the putatively interfering residues appeared to be conserved among the family of Fic-enzymes that

carry the motif N- (class II) or C-terminal (class III) to the Fic-motif. Crystallographic analysis of NmFic (class III) from *Neisseria meningitidis* in complex with AMP-PNP revealed the mechanism of autoinhibition (**Figure 3**). The conserved glutamate of α_{inh} engages with the second arginine of the Fic-motif and thereby sterically and electrostatically interferes with the γ–phosphate of bound ATP. This enforced mode of ATP binding would not allow for AMP-transfer since the position that is in-line with the Pα-Oα bond for attack by the substrate's nucleophilic side chain is occluded by other side chains. Importantly, the researchers demonstrated that substitution of the conserved glutamate by a glycine relieves the autoinhibition which provided a means to study the function of Fic-enzymes. The concept of α_{inh} mediated autoinhibition proved to be true for most Fic-proteins but not for CdFic from *Clostridium difficile* that displays an alternative mode of ATP binding due to structural changes within the ATP binding pocket.[49,50] In CdFic, a position that in Fic-enzymes is usually occupied by a hydrophobic residue is replaced by an arginine which binds to the α–phosphate and thereby reorientates ATP in a conformation compatible for AMP-transfer.[49] While the molecular basis for autoinhibition is well understood, its relief in physiology (and not by mutagenesis of the conserved glutamate within α_{inh}) remains, in great parts, unclear. In 2016, a study suggested that the enzymatic activity of class III Fic-enzymes (herein NmFic) is inversely regulated by two mechanisms, namely tetramerization and autoAMPylation.[51] While the tetrameric form of NmFic is inactive, concentration-dependent dissociation to dimers/monomers enables autoAMPylation in *cis.* Since the modified residue is a buried tyrosine within α_{inh}, its modification leads to the partial unfolding of α_{inh} and thus relief of autoinhibition.

Another layer of regulation of AMPylation represents the deAMPylation of AMPylated substrates.[52] While phosphodiesterases are able to cleave AMP from AMPylated substrates *in vitro*, the physiological role of phosphodiesterases as deAMPylases yet remains to be determined.[13] Meanwhile the deAMPylation activity of Fic-enzymes was discovered.[16,53,54] Surprisingly, the α_{inh} that hitherto was considered as an inhibitory module that simply impairs productive ATP binding was shown to confer deAMPylation activity towards its dedicated substrates.[16,53] Of note, hydrolysis of the phosphodiester bond depends on the catalytic histidine, demonstrating that the same active center is used for catalysis of a different reaction. Moreover, change of the oligomeric state of the human and Drosophila Fic-enzyme FICD/dFic was demonstrated to shift the reaction specificity from AMPylation to deAMPylation and vice versa.[18,53] Dimeric FICD predominantly acts as deAMPylase while monomeric FICD is able to AMPylate its substrate despite the integrity of the α_{inh} and still retains deAMPylation activity. The ability of Fic-enzymes to both modify and demodify their targets seems to be a wide-spread phenomenon since to date it has been also observed for the toxin doc (phosphorylation/dephosphorylation) and the class III Fic-enzyme EcFic from *Enterococcus faecalis* (AMPylation/deAMPylation).[45,54] Only recently, it has been reported that the dual

enzymatic activity of Fic-enzymes may, in addition to oligomerization, be attenuated by Mg^{2+} -and Ca^{2+}-binding to the catalytic site.[54] While Mg^{2+}-binding is known to be critical for AMPylation, the authors demonstrate by biochemical and structural studies that Ca^{2+} is not a suitable cofactor for catalyzing AMP-transfer. On the contrary, deAMPylation activity is supported by both Mg^{2+} and Ca^{2+} ions. These differential effects of metal ions on AMPylation and deAMPylation constitute a piece in the puzzle of regulation of the dual Fic-enzyme activity. Importantly, the described differential effects differ in regards to the human Fic-enzyme FICD/HYPE (see chapter 1.2.4).[54]

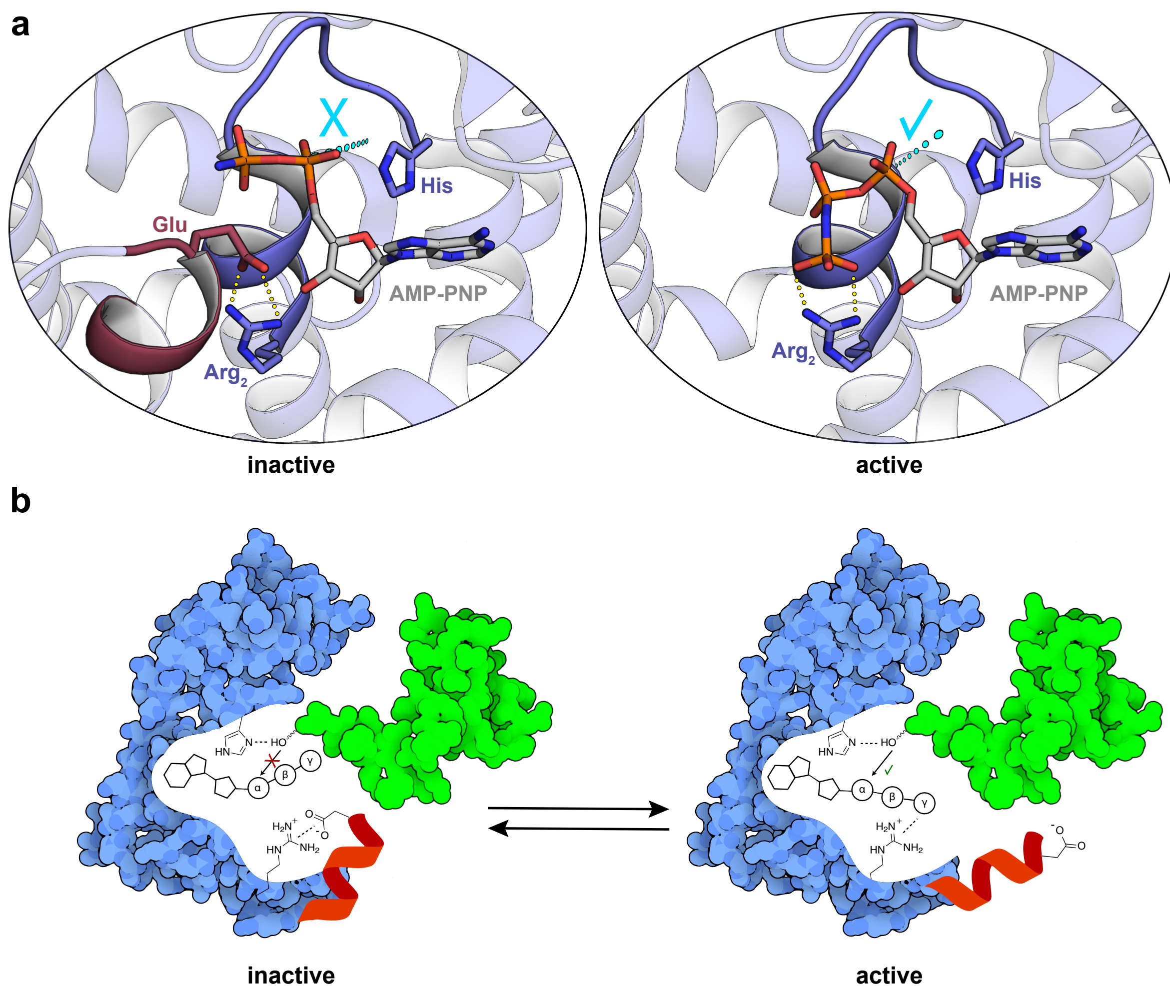

Figure 3 | Autoinhibition of Fic-enzymes by the α_{inh}. a) *The class III Fic-enzyme NmFic (purple) is autoinhibited by a C-terminal α_{inh} (red) that displays a conserved glutamate residue (left panel, PDB 3S6A).[28] The conserved glutamate engages with the second arginine of the Fic-motif (highlighted in purple) and thereby sterically and electrostatically interferes with the competent orientation of the phosphates. As indicated by the cyan dots the position of the nucleophile that is required for an attack in-line with the Pα-Oα bond is obstructed by the Fic-enzyme. The γ-phosphate is flexible and therefore not shown. Deletion of the inhibitory helix leads to competent ATP binding (right panel, PDB 3SE5). The second arginine of the Fic-motif engages with the γ-phosphate and the α-phosphate is optimally positioned for an S_N2 nucleophilic substitution.* ***b)*** *Schematic representation of the derived model for inhibition by the α_{inh}.[28,30] Fic-enzymes (blue) are autoinhibited and become activated upon dislocation of the α_{inh} (red) that enables competent ATP binding.*

While both AMPylation and deAMPylation were also attributed to enzymes with a DNA polymerase β-like fold, it appears that these enzymes do not use the same catalytic site for both

chemical reactions. For instance, AMPylation mediated by the *E. coli* DNA polymerase β-like fold AMPylase GS-AT was also shown to be reversed by the same protein, yet by a separate domain and with distinct mechanistic characteristics:[6–9] While the C-terminal domain exclusively acts as AMPylase, the separate N-terminal domain (also DNA polymerase β-like fold) catalyzes deAMPylation via phosphorolysis rather than hydrolysis. The dual enzymatic activity of GS-ATs is governed by binding of both metabolites and the regulatory protein PII that itself is subject to covalent modification with UMP thereby altering its downstream signaling. The binding of the protein PII to GS-AT stimulates AMPylation activity, whereas UMPylated PII stimulates deAMPylation.

Furthermore, AMPylation of the small GTPase Rab1b by the Legionella effector DNA polymerase β-like fold enzyme DrrA/SidM was also shown to be reversible.[55,56] During infection of host cells, Legionella secretes many effector proteins to manipulate the host cell.[57] By temporal regulation of effector secretion (early phase: AMPylase DrrA; late phase: deAMPylase SidD) target AMPylation is controlled.[55] Interestingly, the structure of the deAMPylase SidD resembles a metal-dependent phosphatase domain rather than a Fic or DNA polymerase β-like fold.[29] In a recent twist, the Legionella AMPylase DrrA/SidM was shown to require allosteric activation by its AMPylation substrate Rab1b prior to AMPylation of a second Rab1b molecule binding to its catalytic site.[58] The allosteric binding event results in remodeling of the catalytic site to its active conformation. This mechanism is hypothesized to prevent unspecific AMPylation and thereby control cytotoxicity upon Legionella infection.

1.2.3 Functional consequences of AMPylation

Herein, the functional consequences of AMPylation are highlighted. Since the consequences of AMPylation by the metazoan Fic-enzyme FICD are discussed under section 1.3.3, this section is divided into AMPylation in the context of bacterial infection and bacterial homeostasis.

Prior to the rediscovery of AMPylation in 2009, the manipulation of small GTPases by intercellular pathogens was already described as an important process for successful infection of host cells.[59–61] Small GTPases are considered as molecular switches that can be activated upon GTP binding (mediated by GTP exchange factors, GEFs) and inactivated upon hydrolysis (stimulated by GTPase activating proteins, GAPs).[62] These molecular switches govern numerous processes such as vesicular trafficking (Rabs), nuclear protein import/export (Ran), and cytoskeleton dynamics (Rho) which makes them hot targets for bacterial toxins.[14,63] For instance, in the course of Legionella infection, the AMPylase DrrA/SidM is secreted to the host cell which early on was associated with the activation of the small GTPase Rab1b in order to establish and maintain a specialized bacteria-generated subcompartment, the Legionella-containing vacuole (LCV).[59]

However, the important role of AMPylation in Legionella infection was not appreciated until 2010.[12] DrrA AMPylates Rab1b at a conserved tyrosine (Y77) residue within switch II, an important hub for both regulatory proteins and downstream effectors. Importantly, AMPylation directly forces Rab1b to its active conformation, regardless of the bound nucleotide (GDP or GTP).[64] In addition, AMPylation in switch II efficiently disrupts interactions with both effectors and proteins that are known to inactivate Rab1b (e.g. GAPs).[12] Hence, upon AMPylation, Rab1b is locked in its active state that is contributing to the maintenance of the LCV.[12,59]

Furthermore, two other toxins of bacterial pathogens are known to disrupt cellular signaling by AMPylation of small GTPases. VopS from *Vibrio parahaemolyticus* and IbpA from *Histophilus somni* target Rho GTPases such as RhoA, Rac1, and Cdc42.[10,13] Despite the structural similarity of VopS and IbpA, VopS AMPylates a threonine residue (T35 in Rac1) whereas IbpA modifies a tyrosine residue (Y32 in Rac1) that both lie within the switch I region. In contrast to switch II AMPylation in Rab1b, AMPylation of Cdc42 on switch I only mildly affects the molecular properties in a direct manner.[64] Like switch II, switch I represents an important interface to communicate with effector proteins.[62] AMPylation of switch I disrupts this communication and thus inhibits the formation of cytoskeletal structures. In living cells AMPylation of Rho GTPases results in cell rounding, indicating rapid cytoskeleton collapse.[10,61,65] Interestingly, inhibition of Rho GTPases by AMPylation also subverted other host signaling pathways such as the immune response by inhibition of the NFκB signaling.[66] Only recently, IbpA was reported to AMPylate also membrane-bound host receptors, that are involved in endocytosis and iron metabolism suggesting that further pathways are manipulated by pathogen mediated AMPylation.[67]

The pathogen *Bartonella henselae* secretes a multitude of effectors (called Beps) many of which comprise a Fic-domain.[27] So far, only little is known about the role of Beps in pathogenicity. While the effector Bep2 is known to AMPylate the filament protein vimentin, the biological meaning of which is unclear.[68] The effector BepA appears to AMPylate host cell proteins of 40 kDa and 50 kDa and was recently shown to AMPylate the protein p130Cas.[36,67] Since the protein p130Cas is involved in integrin signaling, its manipulation may contribute to bacterial uptake as demonstrated for other pathogens.[69] Furthermore, the effector Bep1 of *Bartonella rochalimae* was shown to specifically AMPylate Y32 of the Rho GTPase subfamily of Rac GTPases, but in contrast to VopS and IbpA, not the related subfamily of Cdc42 and Rho GTPases.[70] The specificity is suggested to be part of a fine-tuned strategy to evade the innate immune response by altering Rac signaling and at the same time avoiding collateral damage that would result from global AMPylation of Rho GTPases.[70]

It should be mentioned that Fic-enzymes are able to mediate pathogenesis beyond the instrument of AMPylation. Since these mechanisms are beyond the scope of this section, they are only briefly

outlined. The Legionella effector AnkX phosphocholinates its substrate Rab1b on S76 within switch II.[32] In contrast to Rab1b AMPylation on Y77, phosphocholination does not overrule the structural changes dictated by the bound nucleotide.[64] Hence, the toxicity of phosphocholinated Rab1b is mainly driven by its altered binding properties towards effectors.[32,42] The plant pathogen *Xanthomonas campestris* transfers UMP to the two kinases BIKI and RIPK, thereby blocking their activation which ultimately hampers the innate immune response.[40] Moreover, the Fic-enzyme AvrB from *Pseudomonas syringae* and the Bartonella effector BepC mediate toxicity to their hosts independent from catalytic activity.[71,72]

The importance of AMPylation in bacterial homeostasis was demonstrated in several case studies. The *E. coli* GS-AT AMPylates glutamine synthase as a response to high cellular nitrogen levels and thereby inhibits its enzymatic activity.[9] Structural studies on the glutamine synthase suggest that enzymatic activity of glutamine synthase may be reduced due to restricted access to the catalytic site upon AMPylation.[73–75] Furthermore the role of bacterial toxin/antitoxin modules that belong to the class I Fic-enzymes was clarified with the identification of their dedicated targets. Two independent studies identified bacterial type II topoisomerases (DNA gyrase and topo IV) as AMPylation substrates of the toxin FicT of *Yersinia enterocolitica* and several homologs.[76,77] The AMPylation site was mapped to the ATP binding pocket which results in impaired ATPase activity.[77] Inactivation of the topoisomerase II ultimately results in altered DNA topology, activation of cellular response to DNA damage and growth arrest.[76,77] While toxin/antitoxin systems are often involved in processes such as plasmid addiction, persister formation, and bacteriophage defense, the biological role of the Fic-related toxin/antitoxin system remains elusive.[27,78] Similar to FicT, the class III Fic-enzyme NmFic was demonstrated to modify DNA gyrase at the same residue as FicT, but also here the physiological role is unclear.[51]

1.2.4 The metazoan Fic-enzyme FICD/HYPE

Metazoans encode no more than one single Fic-enzyme, FICD/HYPE.[79] To date most research was conducted on the FICD orthologues of *Drosophila melanogaster*, *Caenorhabditis elegans,* and *Homo sapiens*.[80] FICD is ubiquitously expressed, albeit at very low levels.[13,81] FICD is a homodimeric protein and consists of several domains of which one exhibits the conserved Fic fold bearing the canonical Fic-motif as well as the α_{inh}-module (**Figure 4**).[37] The Fic domain is located at the C-terminus and constitutes the interface for dimerization. The Fic domain is connected to two TPR motifs via a helix that serves as a linker.[37] The N-terminal sequence of FICD is predicted to encode a type II transmembrane protein. The Fic domain and the TPR motifs are separated from the transmembrane domain by a long amino acid stretch that is predicted to be largely unstructured (**Figure 4**).[37] FICD has been reported to locate to the endoplasmic reticulum (ER) with the catalytic Fic domain faced towards the ER lumen.[34,82–84]

The ER-resident chaperone BiP represents a *bona fide* substrate of FICD and its AMPylation has been studied both *in vitro* and *in vivo* by several research groups.[15,33,34,81] However, considering the conserved sequence of the substrate BiP, it is surprising that FICD orthologs appear to target different residues within the same target protein. For instance, the *C. elegans* FICD homolog (Fic-1) was reported to AMPylate T176 in HSP-3 (BiP orthologue),[81] whereas human FICD was reported in different studies to AMPylate residues S365, T366, and T518.[15,34] Similar to human FICD, its ortholog in *Drosophila melanogaster,* dFic, AMPylates residues T366 and T518.[33,53] Furthermore, the physiological role of BiP AMPylation seem to differ for different organisms. While both human FICD and Drosophila dFic are associated with ER protein homeostasis and ER stress,[15,33,34] Fic-1 does not relate to ER stress, since hyper –or hypo AMPylation did not alter the survival of worms under stress conditions.[81]

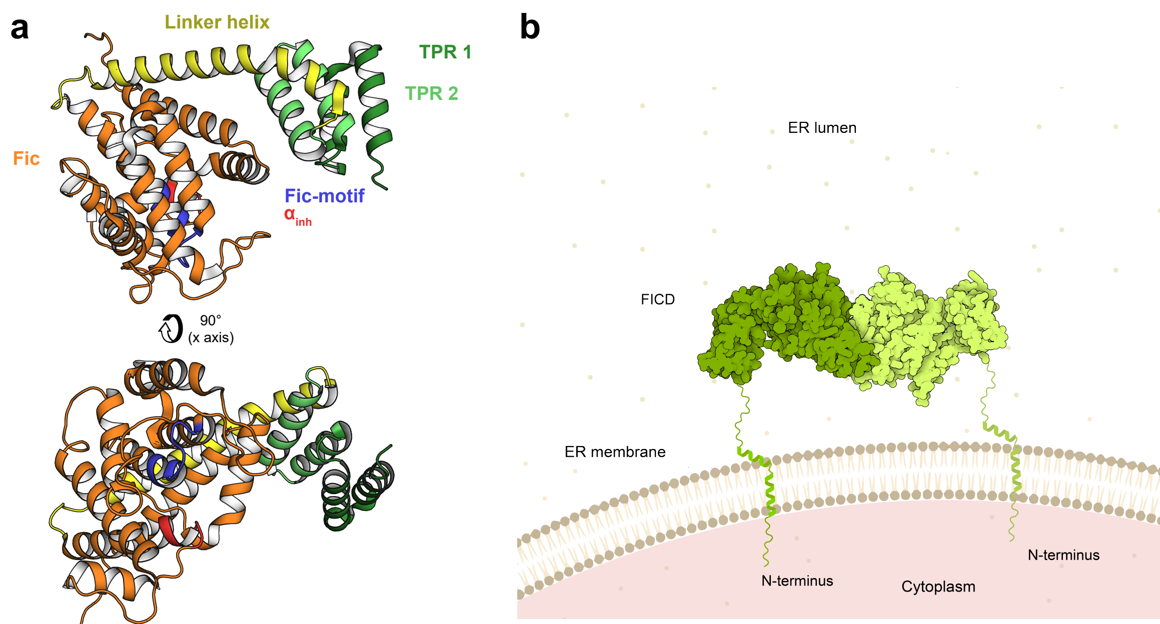

Figure 4 | Structure and topology of the human Fic-enzyme FICD. a) *Ribbon presentation of FICD (PDB 6I7J) comprising the TPR motifs, the linker helix, and the Fic domain. Structural motifs and domains are colored as indicated.* ***b)*** *Topology of dimeric FICD. FICD is anchored to the ER membrane via a transmembrane helix. A structurally flexible linker connects the transmembrane helix to the TPR motifs and Fic domain. This figure was adapted from Fauser et al 2021.[85] (CC BY 4.0).*

Moreover, both human FICD and *C. elegans* Fic-1 have been demonstrated to modify diverse targets *in vitro* such as histones, heat-shock proteins, tubulins, Rho GTPases, and translation elongation factors.[13,15,28,33,34,46,67,81,84,86–88] However, many of the reported targets are cytosolic. While a fraction of *C. elegans* Fic-1 appears to locate in the cytosol, this finding was not confirmed for either human or Drosophila dFic.[81]

As for most Fic-enzymes, the activity of FICD is autoinhibited by the α_{inh}-module. FICD is known to modify itself with an AMP moiety on at least three residues S79, T80 (both within the

unstructured region), and T183 (within the linker helix).[34] However, unlike class III Fic-enzymes, the automodification sites do not locate to α_{inh}, which puts a putative regulatory function of automodification in FICD in question.[51] It was demonstrated that FICD WT is not simply inactive, but a potent deAMPylase,[16] whereas an upon point mutation enforced monomeric FICD (and dFic) represents an AMPylase, even in the presence of an intact α_{inh}-module.[18,53] Of note, monomeric FICD still retains deAMPylation activity (2-fold reduction in comparison to WT).[18] In a recent study it was demonstrated that also dimeric FICD WT retains AMPylation activity (19x-fold less in comparison to monomeric FICD).[18] By adding excess of a constitutive (disulfide-linked) dimeric, catalytically inactive FICD to the AMPylation reaction, the authors managed to trap *in situ* produced AMPylated BiP, thereby prevented its deAMPylation, and thus were able to exclusively quantify AMPylation of BiP.[18] Analysis of the crystal structure of dimeric FICD suggested a close communication of the dimer interface and the α_{inh}-module.[18,37] This notion was supported by *in vitro* experiments assessing the consequences of disrupting the corresponding hydrogen bond network by alanine substitutions. While FICD relay mutants proved to be still dimeric, they exhibited notable AMPylation activity.[18] Structural studies on both dimeric and monomeric FICD revealed that the overall structure of FICD is not changed upon monomerization, yet the mode of ATP binding and the orientation of the conserved glutamate within α_{inh} is changed.[18] While ATP in dimeric FICD does not allow for an attack in line with Pα-Oα due to steric clashes, the ATP's conformation in monomeric FICD is compatible with the nucleophilic attack by the substrate's nucleophilic side chain. The authors conclude, that monomeric FICD binds ATP in a catalytically competent manner as enhanced flexibility of the α_{inh} allows a conformation of the side chain of the conserved glutamate compatible with productive ATP binding. In addition, the authors demonstrated retrograde communication of the active site to the dimerization interface. Specifically, ATP shifts the equilibrium of FICD towards its monomeric state, whereas ADP favors the formation of dimers.[18] Another layer of regulation constitutes the availability of Ca^{2+} and Mg^{2+} ions.[54] AMPylation and deAMPylation generally depend on divalent cations such as Mg^{2+} or Mn^{2+}.[4,54] The suitability of Ca^{2+} ions for FICD mediated AMPylation has been demonstrated.[33,34,54] While other AMP-transferases efficiently use Ca^{2+} ions as cofactors for deAMPylation, Ca^{2+} binds to FICD in a non-productive manner, effectively competing against productive Mg^{2+} binding.[54] Hence, the concentrations of Ca^{2+} and Mg^{2+} ions in the ER lumen constitute another means to fine-tune the dual enzymatic activities of FICD.[54]

1.3 Protein homeostasis in the endoplasmic reticulum

Protein homeostasis describes the regulation and maintenance of a functional ensemble of the proteome. Proteins fold into well-defined three-dimensional structures that allow them to fulfill their function. While this three-dimensional structure is defined in the protein's primary sequence,[1] not all proteins successfully attain their final structure and may be trapped in

intermediate or misfolded states.[89] Accumulation of misfolded proteins can ultimately lead to protein aggregation and cellular toxicity. Chaperones are proteins that bind and stabilize misfolded proteins (also referred to as clients) and thereby prevent their accumulation and aggregation.[89] Often their action is regulated and supported by cochaperones. The endoplasmic reticulum (ER) plays a central role in the synthesis and distribution of secreted, organellar, and membrane proteins. A third of all synthesized proteins in the cell is produced in the ER, underlining the critical role of ER protein quality control for cellular health.[90]

Proteins that are targeted to the ER usually contain an N-terminal signal sequence that after synthesis and cofactor binding (signal recognition particle) stalls ribosomal translation and directs the ribosome to the ER.[91] After binding to the import complex, the nascent polypeptide is cotranslationally translocated into the ER, where the polypeptide is assisted in folding by diverse chaperones and cochaperones. The calnexin/calreticulin system represents a modular protein folding platform that recruits cochaperones such as protein disulfide isomerases or peptidylprolyl isomerases.[92] The export of immature proteins to the Golgi is prevented by monitoring the folding process via a sugar code.[93] Furthermore, other chaperones, the Hsp90 homolog Grp94 and the Hsp70 homolog BiP are present in the ER.[94] Despite the redundancy in ER protein quality control, internal and external stressors can perturb the balance between chaperones and their clients, which gives rise to a regulatory intervention by the cell, called the unfolded protein response (UPR).[95]

1.3.1 The unfolded protein response

The unfolded protein response describes a global cellular program that is launched when the burden of unfolded proteins exceeds the ER folding capacity.[95] Via three separate branches the signal of ER stress is transduced to several effectors, that ultimately restore ER protein homeostasis via several measures (**Figure 5**). The first signal transducer is IRE1 (inositol requiring enzyme 1), which is highly conserved among eukaryotes.[96,97] IRE1 is a single-pass transmembrane protein that comprises a luminal as well as a cytosolic kinase and an RNAse domain.[97] Upon ER stress, the luminal domains of IRE1 dimerize and thereby induce autophosphorylation of the cytosolic kinase domains.[98] The RNAse domain attains its active conformation, specifically splices its dedicated substrate, and thereby induces translation of the transcription factor XBP1.[99] XBP1 induces the expression of genes that enhance the folding capacity of the ER such as chaperones.[100] In addition, IRE1 is shown to reduce the folding load by directly degrading mRNAs at the translocon, a process that is referred to as regulated IRE1-dependent decay (RIDD).[101,102] Currently, two established models exist that may explain how IRE1 senses the load of unfolded proteins in the ER.[97] First, dimerization and activation are mediated by direct binding of unfolded proteins to the luminal IRE1 domains.[103] Second, at times of low

folding load, BiP is guided by specific cochaperones to IRE1 and thereby prevents its dimerization. Under ER stress, unfolded proteins compete with IRE1 for BiP binding and consequently enable dimerization of IRE1.[104,105] Similar to IRE1, the second transducer PERK is a transmembrane protein and activated via oligomerization and autophosphorylation, enabling subsequent phosphorylation and inactivation of translation initiation factor eIF2α. Thus, global protein synthesis is inhibited, while the expression of a specific subset of genes is elevated, thus promoting ATF4 dependent gene expression of chaperones and regulators of redox homeostasis.[106] The third signal transducer is the transmembrane protein ATF6 which is exported to the Golgi and proteolytically cleaved upon ER stress.[106] The cytosolic domain acts as a transcription factor and activates the expression of ER chaperones.[100]

If cells fail to restore protein homeostasis by activating the UPR and therefore suffer from sustained ER stress, apoptosis is induced as the last step.[95]

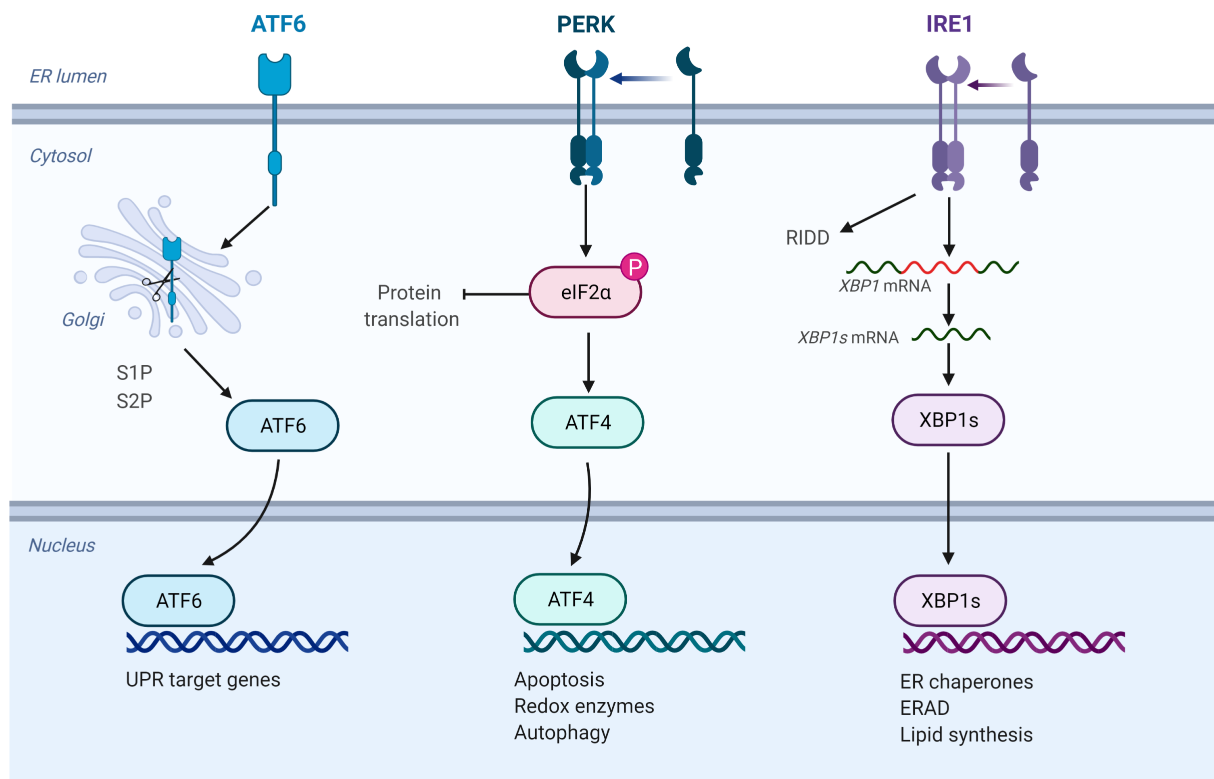

Figure 5 | Schematic representation of the three branches of the unfolded protein response. *ATF6 is transported to the Golgi upon ER stress. Proteolytic cleavage releases the N-terminal domain that acts as a transcription factor. PERK is autophosphorylated and signals via phosphorylation of eIF2α. IRE1 acts via direct degradation of mRNAs at the translocon (RIDD) and adapts gene expression via splicing and activation of XBP1. Created with BioRender.com.*

1.3.2 Structure and function of BiP

The ER-resident chaperone BiP belongs to the class of Hsp70 chaperones that exhibit a characteristic domain structure. They possess an N-terminal nucleotide-binding domain (NBD) and a C-terminal substrate-binding domain (SBD) that are connected by a highly conserved hydrophobic linker (**Figure 6**). The substrate-binding domain is defined by a β-sheet rich structure (SBDβ) and an adjacent α–helical lid (SBDα) at the C-terminus of the protein.[107] Generally, the activity of Hsp70 chaperones is dependent on ATP, and is regulated by Hsp40 cochaperones (also referred to as J-domain proteins JDPs) and nucleotide exchange factors (NEFs). In general, Hsp70s are highly conserved. Since *E. coli* DnaK (Hsp70) and DnaJ (Hsp40) are well studied, they often serve as a reference for other Hsp70 systems and mechanistic insights gained from DnaK/DnaJ often apply to other Hsp70s, too.[108]

BiP has many functions in the ER. Importantly, it acts as a general chaperone by binding and stabilizing (partially) unfolded proteins.[89] It binds to a wide variety of polypeptides that expose preferably hydrophobic stretches of 5-7 amino acids.[109–111] As mentioned in the previous chapter, it is also involved in the activation of the unfolded protein response.[104,105] Furthermore, BiP contributes to protein import at the Sec61 translocon[112] and export of misfolded proteins[113] and, in addition, is important for the maintenance of the Ca^{2+} gradient between the ER and the cytosol.[114,115]

The functions of BiP are governed by its ATPase cycle (**Figure 6**). The ATP-bound state of BiP is structurally well-defined and was in detail analyzed by X-ray crystallography and single-molecule studies.[107,116] In the ATP bound state, the NBD and SBD are docked to each other with the conserved hydrophobic linker being inserted into the NBD. The lid is bound to the NBD and the substrate-binding pocket wide open. The affinity to protein substrates in this state is low with high association and dissociation rates.[117] Upon ATP hydrolysis to ADP, BiP adopts a different conformation: The hydrophobic linker is released from the NBD and the NBD and SBD are undocked.[116,118] The substrate-binding pocket is closed and covered by the lid.[107,119] In the ADP state, the affinity to substrates is high with low dissociation and association rates.[117] The described allosteric control of substrate and nucleotide-binding was intensively studied in *E. coli* DnaK and is likely to apply for BiP due to the high conservation of the proteins.[108,120–122] In particular, the conserved hydrophobic linker that connects SBD and NBD was shown to play a central role in interdomain communication.[123–127]

The ATPase cycle is regulated by cochaperones and nucleotide exchange factors. In the ER, seven J-domain proteins are known.[94] While JDPs differ in their modular structure and their functions, they all share the J-domain that specifically recognizes the NBD of BiP.[128–130] JDPs are considered

to bind clients by adjacent domains, transfer the client to the SBD of ATP-bound BiP, and stimulate ATP hydrolysis, which results in the tight binding of BiP to its substrate.[131] The interplay of Hsp70s with their corresponding Hsp40s results in ultra-affinity of Hsp70s towards their folding clients, which is characterized by the high substrate association rates of BiP:ATP and the low dissociation rates of BiP:ADP.[117,132] Like other Hsp70s, BiP exhibits slow intrinsic ATPase activity[133] that is strongly promoted upon substrate and/or JDP binding.[134] In contrast to other Hsp70s like DnaK, *in vitro* ATP hydrolysis of BiP is stimulated only by peptides and not by unfolded protein substrates.[135] The release of the substrate is coupled to the function of NEFs, since Hsp70s have slow nucleotide dissociation rates and the high Ca^{2+} concentrations in the ER strongly increase the affinity of BiP towards ADP.[136] In the ER, two different NEFs - Grp170 and Sil1 - accelerate nucleotide exchange of BiP and thus reestablish BiP:ATP with high substrate dissociation rates. At this point, the cycle is closed and another substrate can enter the cycle.

The chaperone activity of BiP is adapted to the requirements by several means. As mentioned in the previous section, the UPR is launched in response to a high burden of unfolded proteins in the ER and results in increased chaperone activity by higher expression levels of BiP.[95] In addition to this mid-term response, BiP is regulated on a shorter time scale by two other mechanisms: First, a portion of BiP is stored as inactive oligomers that are recruited upon ER stress.[137,138] Second, BiP is reversibly modified with AMP, thus attenuating its chaperone activity.[15]

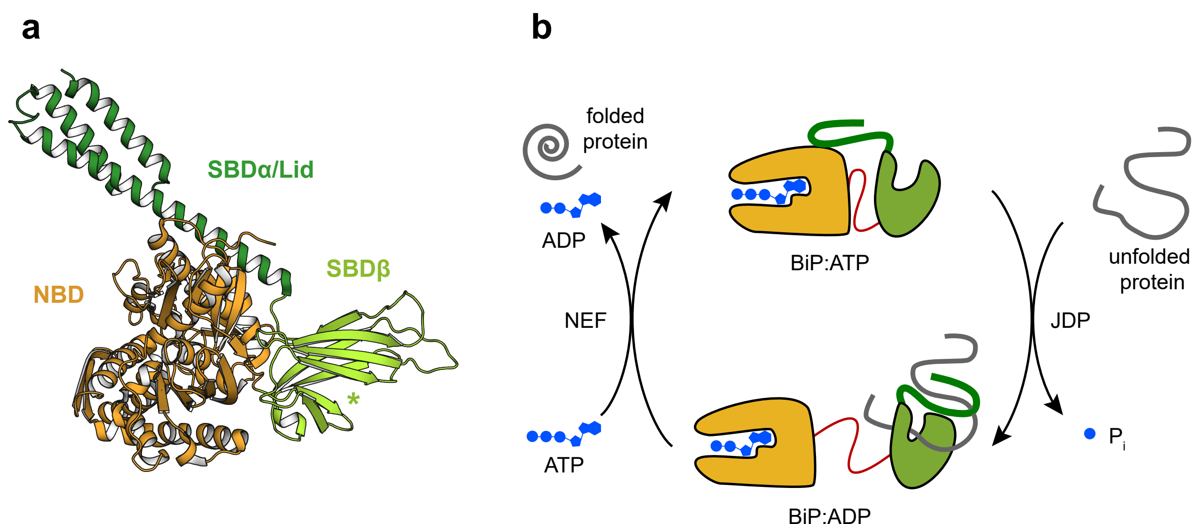

Figure 6 | Structure and function of BiP. a) *The structure of BiP in its ATP-bound state (PDB 5E84). The domains are colored as described and the substrate-binding pocket is indicated with a star.* ***b)*** *Schematic representation of the ATPase cycle of BiP. The colors are encoded as in a). The conserved linker is displayed in red and the nucleotides in blue. In the ATP-bound state, the NBD and SBD are docked to each other and the substrate-binding pocket is open. In the ADP-bound state, the two domains are undocked, the substrate-binding pocket is closed and covered by the lid.*

1.3.3 The (de)AMPylation of BiP as "small unfolded protein response"

Already in the 1980s, BiP was described to be subject to posttranslational modification.[139,140] While the identity of this PTM was never directly determined, BiP was observed to be metabolically labeled with ^{3}H-adenosine as well as ^{32}P-phosphate.[139,140] In addition, the modified species exhibited a lower isoelectric point (pI).[140,141] These observations led to the hypothesis that BiP may either be phosphorylated or ADP-ribosylated. The site of modification was mapped to the SBD.[142] Furthermore, the modification was thought to inactivate BiP, since it correlated inversely with ER folding load[141–143] and modified BiP was found to be free of folding clients.[139,142]

Very early on in the newly revived AMPylation field, FICD-mediated AMPylation was phenotypically related to the integrity of both visual neurotransmission and eye morphology in Drosophila.[53,83] Meanwhile, these observations have been linked to FICD's ability to covalently modify BiP with AMP.[33,53,144] The AMPylation site of BiP was mapped to T366[33,34] and T518[15,17,88], of which the latter seems to be the primary AMPylation site in humans. AMPylation of BiP on T518 is considered as inactivating modification, since BiP^{AMP} displays reduced basal ATPase activity, weakened substrate interaction, and resistance to JDP stimulated ATP hydrolysis *in vitro*.[15,17] Moreover, AMPylation of BiP strongly shifts the conformational equilibrium towards its monomeric, domain-docked conformation even in absence of nucleotide or presence of ADP.[17]

Consistent with these observations, cellular levels of AMPylated BiP are high at normal growth conditions (approx. 15% of total cellular BiP[142]) and are reduced upon ER stress.[15,33,34] Decreasing protein influx to the ER by blocking global translation with cycloheximide leads to enhanced levels of BiP^{AMP}.[15,33] Overexpression of constitutively active FICD promotes the formation of inactive BiP^{AMP}, which leads to induction of the UPR via the PERK branch and ultimately to apoptosis.[15,34] In agreement with this, tissue-specific overexpression of constitutively active FICD in flies resulted in defects in eye morphology that were more pronounced upon silencing of IRE1 and PERK branches.[144] Together, AMPylated BiP is considered to represent a storage form of excess BiP, thus preventing futile ATP consumption and inefficiency in regards to protein secretion in times of low folding load.[145] At the onset of ER stress, this storage pool of BiP^{AMP} is rapidly deAMPylated and recruited to restore ER homeostasis. Hence, BiP-AMPylation-deficient cells (by knock-out of FICD) were shown to have enhanced buffer capacity of the ER in regards to unfolded protein load.[15]

It has been demonstrated *in vitro* that dimeric FICD deAMPylates, whereas monomeric FICD AMPylates BiP (**Figure 7**).[16,18,53] Despite considerable efforts, the determinants for the oligomeric and thus functional switch have not yet been identified. While this question remains to be addressed, two mechanisms were recently identified that allow fine-tuning of the activity of

dimeric and monomeric FICD (**Figure 7**). First, high Ca^{2+} concentrations inhibit FICD mediated deAMPylation of BiP.[54] This observation fits well with the physiological situation in the ER, where disruption of Ca^{2+} homeostasis causes ER stress, which would require recruitment of BiP.[146] High Ca^{2+} levels, however, indicate normal ER conditions, where AMPylation of BiP might be favored over deAMPylation. Second, FICD binding to ATP favors monomerization, whereas FICD binding to ADP favors dimerization.[18] In times of ER stress ATP/ADP ratio might be reduced due to enhanced chaperone activity and thus favor the recruitment of BiP by deAMPylation.

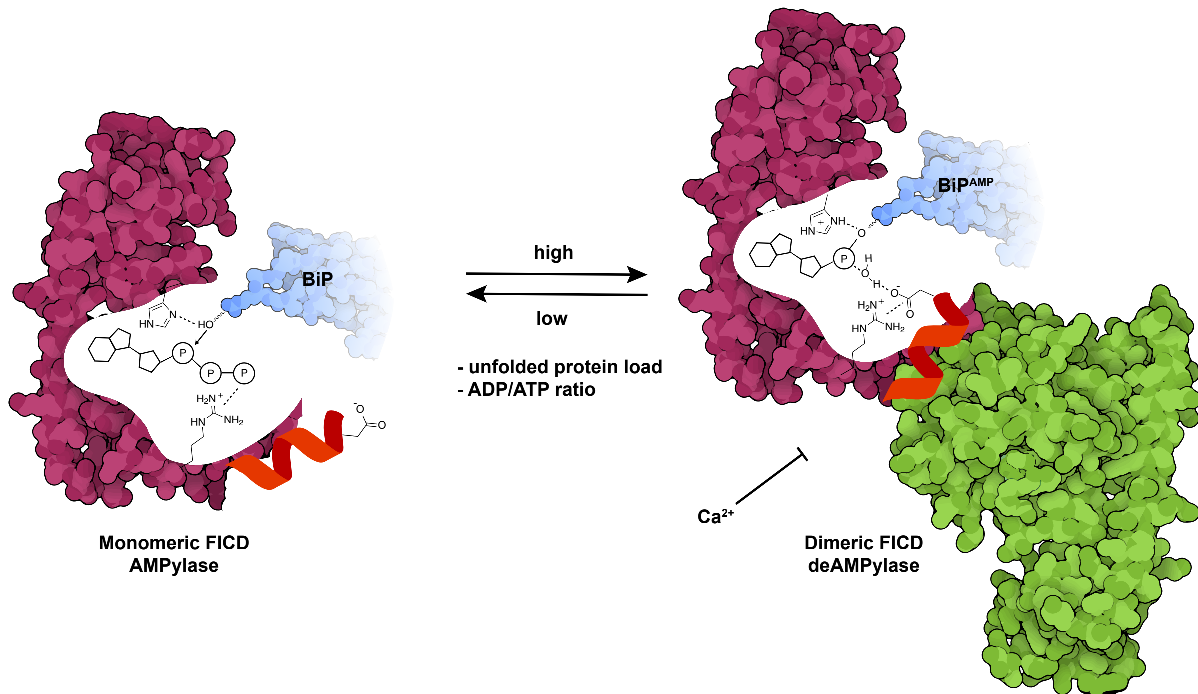

Figure 7 | Proposed model of FICD regulation. *Monomeric FICD is AMPylation active. The* α_{inh} *is displaced thus permitting productive ATP binding. In dimeric FICD the conserved Glu of* α_{inh} *engages with an Arg of the Fic-motif and thereby obstructs productive ATP binding. On the contrary, glutamate orientates a water molecule for the attack at the α-phosphate. The catalytic His provides a proton to threonine, thus favoring cleavage of the phosphodiester bond. Oligomerization is attenuated by the unfolded protein load and ADP/ATP binding. Non-productive binding of* Ca^{2+} *to the active site competes with* Mg^{2+} *binding and inhibits deAMPylation.*

1.4 Strategies for identification of AMPylation substrates

Suitable methods to reliably detect AMPylation within proteins are a prerequisite for the identification of AMPylation substrates/targets. To this end, several methods were established that allow the detection of AMPylation.[4,29] Commonly, radioactively labeled ATP (^{32}P-α-ATP) is used as a cosubstrate to detect AMPylation *in vitro*.[6,10,13] Another technique relies on the use of antibodies, that specifically recognize the AMP moiety on a peptide backbone. While previous polyclonal antibodies successfully detect AMPylation *in vitro* e.g. via western blotting, they suffer from limited sensitivity and bias towards the AMPylated peptide backbone they were raised against.[147,148] A very recently developed antibody, however, proved advantageous, since it was demonstrated to detect diverse AMPylated proteins with high sensitivity.[149] AMPylation is easily

identified via intact mass spectrometry by a mass increase of 329 Da.[10] Furthermore, mass spectrometry (LC-MS/MS) can be used to map the site of modification by detection of characteristic AMP fragments upon collision-induced dissociation.[150,151] While the value of the described methods is mostly restricted to the characterization of known targets, mass spectrometry-based methods are in principle suitable to identify novel AMPylation targets within cell lysates.

Since the complexity of lysates and the low abundance of AMPylation substrates is a great challenge for target identification, it often requires enrichment of AMPylated proteins prior to mass spectrometric analysis. While AMP-specific antibodies in principle permit enrichment of AMPylated proteins, their suitability in this regard has yet to be demonstrated. Meanwhile, ATP derivatives (also referred to as probes) have been developed that allow click-chemistry based enrichment of AMPylation substrates.[152–154] To this end, ATP analogs were developed that either bear a propargyl moiety at the N6 amine of the adenine base or an azide moiety at the C2 of the ribose sugar. ATP derivatives that were modified with an azide at C8 of the adenine base were not transferred by Fic-enzymes.[155] In a lysate environment, the exogenously provided AMPylase transfers the AMP derivative to its dedicated substrate. In a subsequent click-reaction, the modified protein can be equipped with a fluorophore or an affinity tag that is used for enrichment (**Figure 8**).[81,88,152,153,155] Based on this approach, DNA gyrase and topo IV were identified as targets of bacterial class I Fic-enzymes.[77] Furthermore, the technology was extended to an array format where novel targets can be systematically identified.[152] The microarray is spotted with the cDNA of potential targets that are fused to an affinity tag. After *in vitro* transcription/translation, the produced protein is kept in place by an immobilized tag-specific binding reagent. Upon addition of the AMPylase in question and N6-propargyl ATP, the putative target is modified. In a subsequent click-reaction, the modified substrates are labeled with a fluorophore, thus permitting a fast and sensitive readout. While the screening of targets in an array format is an artificial setup and restricts the experimenter to the scope of provided cDNA library, lysate-based methods suffer from other disadvantages. For instance, the competition of the ATP derivative against endogenous ATP in cell lysates may effectively reduce the sensitivity of the method. A different approach on target identification is based on the use of different stable isotope-labeled ATP that yield a characteristic mass shift upon AMP-transfer.[68] While reducing competition against endogenous ATP, the authors accepted in return the high complexity of the mass spectra since no specific enrichment was possible. The method was successfully applied for the identification of vimentin as an AMPylation substrate of Bep2. The physiological relevance, however, remains unclear.

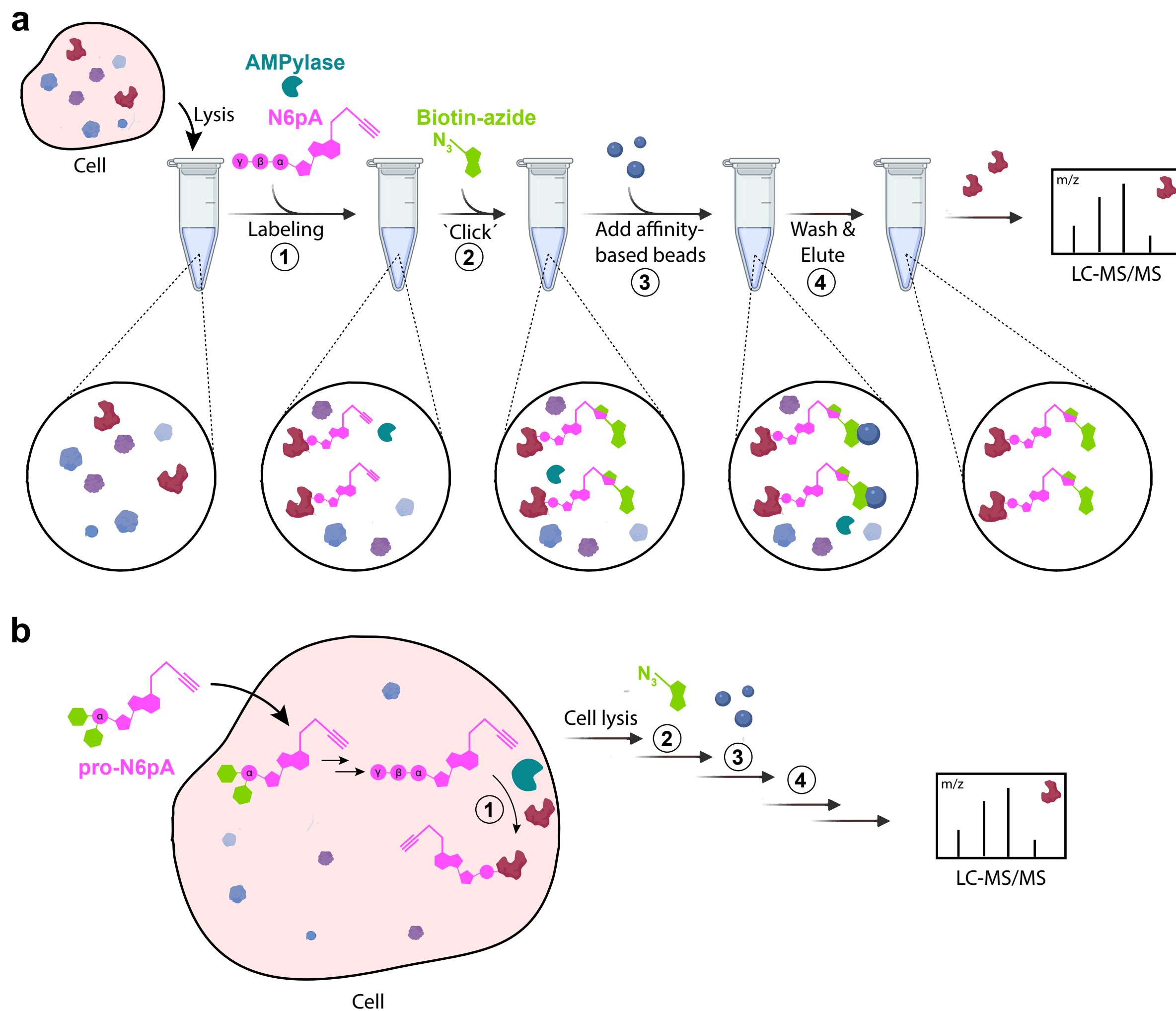

Figure 8 | Strategies for identification of AMPylation substrates. a) *Cell lysates are treated with the AMPylase and an ATP analog (1) that carries a propargyl moiety at the N6 atom (pink, N6pA). The transferred ATP analog reacts with biotin-azide (green) via copper-catalyzed azide alkyne cycloaddition ("Click" reaction) (2) enabling subsequent enrichment with streptavidin-coated beads (3). The beads are washed and the modified proteins eluted (4) for analysis via LC-MS/MS. The AMPylase is colored in petrol and the corresponding AMPylation substrate in red.* ***b)*** *The cell-permeable pronucleotide probe (pink/green, pro-N6pA) is taken up by the cell and converted to a transferable N6pA that is transferred in cellulo. The numbered steps for enrichment refer to panel a). Color coding as in a). Created with BioRender.com*

Until recently, systematic identification of novel AMPylation substrates was restricted to AMPylation events that occur in a lysate or *in vitro* environment since nucleotide derivatives are negatively charged and therefore not cell-permeable. It is anticipated that the disruption of cellular compartmentalization potentially results in the identification of non-endogeneous substrates. In a recent study, researchers addressed this issue by developing a pronucleotide probe.[156] Importantly, the probe is cell-permeable since it consists of a clickable N6-propargyl-equipped AMP in which the charges of the α-phosphate are masked. Within the cell, the probe is cleaved and phosphorylated to its triphosphate state, thus representing an intact cosubstrate for AMPylation reactions (**Figure 8**). The AMPylated substrates are enriched and analyzed via LC-MS/MS. While this approach is very powerful in the systematic identification of AMPylation substrates in the living cell (the AMPylome), the probe competes against high levels of cellular

ATP. Furthermore, the assignment of the corresponding AMPylase requires additional experiments. Nevertheless, this approach provides a valuable control to verify other reported targets, whether they represent pure *in vitro* targets or physiological AMPylation substrates.

2. AIMS

The main aim of this thesis was to elucidate the interaction of the human Fic-enzyme FICD and its physiological substrate BiP. While it was suggested that FICD prefers the domain-docked conformation of BiP as a substrate, the molecular parameters governing this specificity remained elusive. Furthermore, the molecular basis for the oligomeric state-dependent switch that drives reaction specificity towards AMPylation and deAMPylation is not yet understood. Since Fic-enzymes exhibit only low affinity to their protein substrates, an approach to covalently link the two proteins was taken. To this end, bifunctional cosubstrate analogs were synthesized by a collaboration partner, Prof. Christian Hedberg from Umeå University, Sweden, and these cosubstrate analogs successfully applied to covalently link FICD to BiP. The covalently linked complex was to be purified and submitted to crystallography and the obtained complex structure to be verified both *in vitro* and *in vivo*.

In relation to this project, the suitability of bifunctional cosubstrate analogs for the identification of novel AMPylation substrates was to be assessed. Recombinant FICD covalently equipped with the cosubstrate analog would serve as a bait for AMPylation substrates in a lysate environment and form a covalently linked ternary complex that can be enriched via affinity-tags. The pull-downs were to be analyzed via LC-MS/MS by a collaboration partner, Prof. Hartmut Schlüter, University Medical Center Hamburg-Eppendorf, and the enriched proteins analyzed for both validation of the concept and identification of novel substrates.

Furthermore, the pull-down concept was to be extended by covalently linking the AMPylation substrates to magnetic nanoparticles, thus bypassing the need for affinity-based enrichment. A covalently bound target would allow harsh washing conditions during the enrichment procedure, reduce unspecific binding and therefore improve the data quality in LC-MS/MS. To this end, recombinant FICD was envisioned to be immobilized on magnetic nanoparticles via Sortase A mediated ligation. The capture of the target via cosubstrate analogs would ultimately covalently link the target to the nanoparticles.

3. MATERIALS AND METHODS

This section gives an overview on key methods and procedures that were used to obtain the data. Individual methods were adapted from Fauser et al. 2021 (CC BY 4.0) (marked with *) and thus detailed information is provided in the methods sections of the respective publication.[85]

3.1 Molecular biology

For each cloning procedure, the primers were specifically designed to match the requirements in terms of melting temperature and length and were purchased from Integrated DNA Technologies, Inc.. The melting temperature was calculated with the online New England Biolabs (NEB) Tm calculator (http://tmcalculator.neb.com/#!/main). The PCR was performed using Q5® polymerase (NEB) and a standard protocol with elongation times of 20-30 seconds/kb and the products were separated via agarose gel electrophoresis (1% agarose). The purification of the PCR products was achieved using the Monarch Gel Extraction Kit (NEB) according to the manufacturer's instructions.

Site-directed mutagenesis of the genes of interest was achieved by blunt-end ligation applying the Q5® Site-Directed Mutagenesis Kit (NEB). The primers design was performed with an online tool, NEBaseChanger (http://nebasechanger.neb.com/).

Insertion of genes or tags to the plasmid of interest was achieved using SLIC (site and ligation independent cloning) or Gibson-Assembly after gel extraction of the PCR product. To this end, all primers were designed with the corresponding complementary base overlap of 30 bp. Gibson assembly was performed with either homemade or purchased Gibson assembly® (NEB) according to the manufacturer's instructions. SLIC was performed by mixing 100 ng vector with the gene insert at a molar ratio of 1:3 to 1:7 in NEB 2.1 buffer. The mixture is incubated for 2.5 min at room temperature upon addition of T4 DNA polymerase (NEB) and subsequently stored on ice for >10 min. Importantly, while Gibson assembly works smoothly even without gel extraction, SLIC requires DpnI digestion (30 min at 37 °C) and subsequent purification via electrophoresis.

All purified and SLIC/Gibson processed PCR products were transformed into (chemically) Mix & Go competent (Zymo Research) *E. coli* Mach1 cells and plated on LB agar supplemented with the corresponding antibiotic for selection. After 16 h single colonies were picked for inoculation of a 5 mL culture. After > 7 h at 37 °C, the liquid culture was harvested and the plasmids prepped via the PureYield™ Plasmid Miniprep System (Promega) and their concentration determined with the NanoDropTM 2000 (Thermo Scientific). The plasmids were stored at -20 °C and their sequence confirmed via Sanger sequencing using the service of Microsynth Seqlab.

3.2 Recombinant protein expression and purification*

Both FICD and BiP were expressed in *E. coli* Rosetta (DE3) Competent Cells (Novagen). The cells were transformed according to a standard protocol including heat shock and 1 h of incubation in liquid SOC medium prior to plating on LB agar supplemented with the corresponding antibiotic. A single colony was picked for starting an overnight culture in 10 mL LB medium at 37 °C. The cells were transferred into 1 L of prewarmed LB medium and grown to OD_{600} 0.45 – 0.6. Protein expression was induced upon addition of 0.5 mM IPTG and the temperature was dropped to 23 °C. After 16 -20 h the cells were harvested by centrifugation at 7000 g for 15 min and washed once with phosphate-buffered-saline before the pellet was flash-frozen in liquid nitrogen and stored at - 20 °C.

For recombinant production and purification, BiP was expressed with an N-terminal 6xHis-affinity tag, whereas FICD expression and purification were only successful with an N-terminal 6xHis-affinity tag next to a larger solubility tag. While dimeric FICD was successfully produced with an N-terminal Halo-/MBP-/GFP-tag, production of monomeric FICD did not tolerate an N-terminal Halo-tag.

Table 1 | Buffers used for the purification of FICD and BiP.

	FICD	BiP
Buffer A	50 mM HEPES-NaOH pH 7.4, 500 mM NaCl, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol	50 mM HEPES-NaOH pH 7.5, 400 mM NaCl, 20 mM imidazole
Buffer B	Buffer A + 500 mM imidazole	Buffer A + 480 mM imidazole
Dialysis buffer	20 mM HEPES-NaOH pH 7.4, 200 mM NaCl, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol	20 mM HEPES-NaOH pH 7.5, 100 mM NaCl* *BiP Δlid is purified with 200 mM NaCl
SEC buffer	20 mM HEPES-KOH pH 7.4, 150 mM KCl, 1 mM $MgCl_2$, 1 mM TCEP 10% (v/v) glycerol	20 mM HEPES-KOH pH 7.5, 150 mM KCl* 10 mM $MgCl_2$ (HKM buffer) *BiP Δlid is purified with 200 mM KCl

Generally, the cells were thawed, resuspended in ice-cold Buffer A, and homogenized. After addition of DNAse I, the cells were lysed via Constant Cell Disruption Systems (Constant Systems Limited) at 1.8 kbar and the protease inhibitor phenylmethanesulfonyl fluoride was added. The cell debris was separated via centrifugation at 50000 g for 30 min. The supernatant was loaded on a Ni^{2+}-NTA IMAC column (Bio-Rad) using the NGC Liquid Chromatography System (Bio-Rad). The proteins were eluted via a gradually increasing amount of Buffer B. The affinity tags were cleaved by tobacco-etch virus protease during dialysis over-night at 4°C. Both TEV and solubility

tags were removed via reverse Ni^{2+}-NTA IMAC. The proteins were subsequently purified via size-exclusion chromatography (SEC) using a Superdex 16/600 75 pg Gel Filtration Column (GE-Healthcare) for FICD and a Superdex 16/600 200 pg Gel Filtration Column (GE-Healthcare) for BiP, respectively.

3.3 Biochemical and biophysical methods

Binary adduct and ternary complex formation*

Generally, binary adduct formation was conducted in the absence of magnesium to prevent premature hydrolysis of the probe by the engineered Fic-enzyme. Usually, a two-fold molar excess of probe is used for binary adduct formation of FICD and the probe. Typically, 50 µM of FICD react with 100 µM thiol-reactive nucleotide derivative in binary adduct buffer (20 mM HEPES-KOH pH 7.4, 100 mM KCl, 1 mM EDTA) for 16-20 h at 23 °C. The ternary complex is formed upon addition of Mg^{2+} and the substrate BiP at a molar ratio of 1:1. Typically, 30 µM of FICD react with 30 µM BiP in AMPylation buffer (20 mM HEPES-KOH pH 7.4, 100 mM KCl, 4 mM $MgCl_2$, 1 mM $CaCl_2$) for 1-2 h at 23 °C.

Phos-tag™ gel electrophoresis*

Binary adduct formation is reliably monitored via intact mass spectrometry or Phos-tag™ gel electrophoresis. Usually, 2-3 µg of protein are loaded on an acrylamide gel with a 4.5% acrylamide stacking gel and a 12% acrylamide resolving gel. The gel is cast according to the manufacturer's instructions. Importantly, the resolving gel is to be degassed prior to addition of ammonium persulfate and contains in addition to ingredients of regular SDS-PAGE 25 µM Phos-Tag™ AAL-107 (NARD Institute Ltd.) and 100 µM $MnCl_2$. The different protein species are separated within 60-90 min at 30 mA.

Intact mass spectrometry*

High-resolution intact mass spectrometry was performed on a Bruker maXis II™ QTOF mass-spectrometer. The proteins are ionized via electrospray and desalted on a monolithic column (Thermo ProSwift RP-4H 50 mm x ID 1 mm) connected to the Bruker Elute LC system at a flow rate of 0.3 mL/min. The proteins are eluted via a gradient of 5% eluent B to 80% eluent B with eluent A representing milliQ H_2O + 0.1% formic acid and eluent B acetonitrile + 0.1% formic acid. Depending on the size of the protein, 0.2 – 0.6 µg of protein were injected into the column. Data analysis was performed with the program Bruker Compass DataAnalysis 5.1 applying the maximum entropy algorithm for deconvolution.

Western blotting*

Initially, the protein samples are run and separated in 12% or 15% acrylamide gels via SDS-PAGE. The proteins are transferred to the membrane (Immobilon-P PVDF membranes, Merck-Millipore) by applying 320 mA for 1.5 - 2 h (V10-SDB Semi-Dry Blotter, Scie-Plas, Cambridge, UK). After transfer, the membranes are washed once with TBS-T and subsequently blocked with Roti®-Block (Carl Roth) at room temperature for 1 h. The corresponding primary antibody is added at the appropriate concentration and the blots are gently shaken at 4 °C for 16 h. Excess antibody is washed from the membranes three times with TBS-T for 10 min before the corresponding secondary antibody is added. After washing, the corresponding secondary antibody-HRP conjugate is added and membranes are subsequently shaken for 1 h at room temperature. Before imaging on the INTAS ECL CHEMOCAM (Intas Science Imaging) the blots are again washed three times with TBS-T. Development of blots is performed using either WesternBright™ ECL-Spray (Advansta) or SuperSignal™ West Dura Extended Duration Substrate (Thermo Scientific). If necessary, antibodies are stripped from the membranes upon addition of Roti®Free Stripping-Buffer (Carl Roth) for 15 min at 56 °C. The membranes are again blocked after six washing steps and the primary antibody added. The membranes are silver-stained upon addition of the colloidal staining solution that requires harsh vortexing for 1 min during preparation (47 mL milliQ H_2O, 2.5 mL 40% sodium citrate dehydrate, 0.4 g of ferrous sulfate heptahydrate, and 0.5 mL 20% silver nitrate).[157] Excess silver is gently washed from the membrane with milliQ H_2O.

ATPase assay

Typically, 1 – 5 µM of enzyme was incubated with 50 - 100 µM ATP in either AMPylation buffer or 20 mM HEPES-NaOH pH 7.4, 100 mM NaCl 1 mM $MgCl_2$ for 20 – 40 min at 25 °C. As internal standard guanosine was added to the ATP stock solution at a molar ratio of 1:2. The reaction was either directly loaded on the column for kinetic measurements or stopped by cooking at 95 °C for 5 min followed by centrifugation at 21000 g for 5 min. The supernatant was analyzed via reverse-phase chromatography under ion-pairing conditions (stationary phase: Prontosil C18, F184PS050, Bischoff Chromatography; mobile phase: 50 mM KP_i buffer pH 6.6, 12% (v/v) acetonitrile, 10 mM tetra-n-butylammonium bromide) on a Shimadzu UFPLC. The absorbance of the nucleotides was detected at 254 nm, the corresponding peaks integrated and normalized to the internal standard.

Analytical size-exclusion chromatography*

In order to assess the complex formation of BiP/BiP^{AMP} and C_H1, 10 µM BiP was incubated with varying concentrations of labeled or label-free C_H1 in presence of 1 mM ADP for 16 h. Fluorescein-labeled C_H1 was produced to analyze binding to BiP. Therefore, C_H1 is incubated with NHS-Fluorescein (Thermo Scientific) at a molar ratio of 1:1 for 1 h at room temperature and

subsequently purified via desalting (HiTrap desalting column, GE healthcare) and dialysis in HKM buffer. Typically, 25 µg of protein are loaded on a Superdex 200 10/300 (GE Healthcare) and the absorbance is measured at 280 nm (unlabeled C_H1) and 496 nm (labeled C_H1).

The binding of monomeric FICD 102-458 and monomeric FICD 187-458 (ΔTPR) to BiP 19-654 T229A T518A is investigated via analytical size-exclusion chromatography. The proteins are incubated for at least 3 hours to allow complex formation using 5 µM FICD and 100 µM BiP in HKM supplemented with 5 mM ATP. Typically, 25 µg of protein are loaded on a Superdex 75 10/300 (GE Healthcare) and the absorbance is measured at 280 nm. The chromatography is performed with HKM buffer as mobile phase and runs at 0.5 mL/min. The runs of the individual proteins serve as a reference in regards to the retention time of uncomplexed protein.

Steady-state ATPase assay

The steady-state ATPase kinetics of BiP are determined using an ATP regenerating system which at the same time allows real-time monitoring of the reaction progress. First, BiP converts ATP to ADP, which is used by the second enzyme pyruvate kinase (PK) to produce ATP and pyruvate from phosphoenolpyruvate (PEP). In a third reaction, the lactate dehydrogenase (LDH) converts pyruvate and $NADH/H^+$ to lactate and NAD^+. Consumption of $NADH/H^+$ serves as a proxy of ATP hydrolysis and is monitored via absorbance measurement at 340 nm. The reaction is set up in HKM buffer with 2 µM BiP, 1 mM ATP, 3 mM PEP, 20 U/mL of each LDH and PK and 250 µM $NADH/H^+$ at a final volume of 30 µL per sample, thus allowing analysis in a low binding 384-well plate (Corning) in TECAN Spark microplate reader (Tecan). Typically, the reaction mixture is preequilibrated at 37 °C, and the reaction started upon addition of the enzyme BiP. Each measurement is taken at least in triplicates. The k_{cat} of ATP hydrolysis is calculated by the following formula:

$$k_{cat} = \frac{m}{\varepsilon_{NADH,340\,nm} * d * c_{BiP}} \qquad \text{equation (1)}$$

with m as the linear slope, $\varepsilon_{NADH,\,340\,nm}$ as molar extinction coefficient of NADH at 340 nm (6200 M^{-1} cm^{-1}), d as the path length in cm (derived from the absorbance of solutions of known NADH concentrations with Lambert-Beer's law), and c as the molar concentration of BiP.

Circular dichroism spectroscopy*

The proteins are first diluted to 0.1 mg/mL in 10 mM potassium phosphate pH 7.5, 150 mM KF, 1 mM $MgCl_2$, to reduce the amount of HEPES and chloride from the protein storage buffer that would greatly absorb in far UV and therefore produce background signals. The spectra are obtained from 185 – 260 nm with a 0.1 mm cuvette at 25 °C. The instrument settings of the

Chirascan CD spectrometer (Applied Photophysics) are 0.5 nm bandwidth, 0.5 s response, and 0.5 nm data pitch. Data analysis is performed with Pro-Data Viewer and the CD spectra background is subtracted. The experiments are performed in triplicates with each replicate representing the mean of three individual measurements.

Fluorescence polarization

In order to monitor deAMPylation of BiP by FICD WT, 6xHis-BiP was first modified with fluorescein-labeled ATP (N6-(6-Aminohexyl)-ATP-6-FAM, Jena Bioscience) by FICD 102-458 E234G and purified via Macherey-Nagel™ Protino™ Ni-NTA Agarose (Thermo Scientific) and dialysis in HKM buffer. The deAMPylation was performed with 17 nM of modified BiP, 1 µM FICD WT and, if desired, 1 mM ADP/ATP. The reaction was started upon addition of FICD WT and carried out at 25 °C in low-binding 384-well plates (Corning). The filter for excitation at 485 nm and emission at 535 nm are selected.

Determination of protein melting point*

For the determination of the melting points a label-free method, nano dynamic scanning fluorimetry (NanoDSF) was applied. The proteins are charged into standard capillaries (nanotemper, #PR-C002) after dilution to 1 mg/mL in HKM buffer. The melting procedure of the instrument, Prometheus NT.48 (nanotemper), was set to a gradient of 1 °C/min starting from 20 °C to 80 °C. The corresponding melting points were determined from the fluorescence ratio 350 nm/330 nm.

3.4 Software

Adobe Illustrator CS4

Adobe Photoshop CS4

ChemDraw 19.1

Mendeley 1.19.4

GraphPad Prism 8.3.1

Image Lab 6.0.1

Microsoft Office Excel 365

Microsoft Office Word 365

OriginLab, 2019b, v9.65

PyMOL 2.3.2

Illustrate[158]

BioRender

4. PUBLICATIONS

This is a publication-based thesis. Each project has been published in international peer-reviewed journals. In the following, the corresponding publications are briefly summarized.

4.1 Specificity of AMPylation of the human chaperone BiP is mediated by TPR motifs of FICD

The article Specificity of AMPylation of the human chaperone BiP is mediated by TPR motifs of FICD *was published in Nature Communications in April 2021 (DOI: 10.1038/s41467-021-22596-0). The author of this thesis, Joel Fauser, performed molecular biology, protein expression, protein and complex purification, crystallization, biochemical and biophysical experiments, analyzed the data, and wrote the manuscript.*

The endoplasmic reticulum is the key interface in the communication of cells with their environment. It is responsible for the production of a vast amount of proteins and ensures their correct folding and quality. Several mechanisms exist to adapt the folding capacity of the ER to the fluctuating load of unfolded proteins.[15,95,137] One mechanism is the FICD-mediated reversible AMPylation of the Hsp70 chaperone BiP, a process that was shown to control chaperone activity.[15,16,33,34] The structural basis of the interaction of BiP and FICD, however, remained elusive due to the transient nature of the enzyme-substrate complex.

Herein, thiol-reactive nucleotide derivatives (TReNDs - produced by Prof. Christian Hedberg, Umeå University, Sweden) are used to covalently link FICD to BiP and trap the transient AMPylation complex (**Figure 9**). The TReNDs are designed with an attenuated electrophile that reacts with a proximal cysteine as nucleophile, thus yielding a stable thioether-linked binary adduct (**Figure 9**). The protein substrate BiP is linked to FICD in a second step in which the covalently bound TReND serves as a cosubstrate for the AMPylation reaction. I initially produced and tested several cysteine substitutions within FICD for both efficient and regioselective formation of the ternary complex. For homogeneity of the produced complex, with a collaboration partner (Prof. Hartmut Schlüter, UKE, Germany) I determined (via LC-MS/MS) and removed the autoAMPylation sites within FICD. I isolated the complex and submitted it to X-ray crystallography. The collected dataset was analyzed by a colleague, Dr. Vivian Pogenberg. I interpreted the complex structure and observed that FICD specifically recognizes the domain-docked conformation of BiP by engagement of the TPR motifs with the conserved hydrophobic linker of BiP that is inserted into the nucleotide-binding domain. In biochemical assays, I confirmed this observation by demonstrating that the domain-docked conformation of BiP is the preferred substrate for both FICD mediated AMPylation and deAMPylation. Furthermore, I identified crucial interactions of the enzyme-substrate complex and validated the interaction

interface both *in vitro* and *in vivo*. Importantly, my biochemical experiments suggest that substrate recognition in the deAMPylation complex would exhibit a similar interaction profile. As the crystallized AMPylation complex of FICD and BiP appeared to be partially undocked, the interaction profile was extended by molecular dynamics simulation (Prof. Martin Zacharias, TUM, Germany) that was guided and validated by my biochemical data. It was shown previously, that unfolded proteins shift the conformational equilibrium of BiP towards its domain-docked conformation. Nevertheless, I found that the binding of unfolded proteins to BiP does not directly regulate AMPylation and deAMPylation. Moreover, I demonstrated that the TPR motifs of FICD are critical for AMPylation of BiP. The AMPylation of other reported FICD substrates, however, differ in this regard. While AMPylation of the eukaryotic elongation factor 1A2 depends on the TPR motifs, AMPylation of uridine 5'-monophosphate synthase is AMPylated by FICD ΔTPR.

In a nutshell, this work dissects a novel mode of interaction of TPR motifs with Hsp70 proteins and demonstrates the suitability of thiol-reactive cosubstrate analogs for structural biology by solving the previously elusive complex of the only human Fic AMPylase and its substrate.

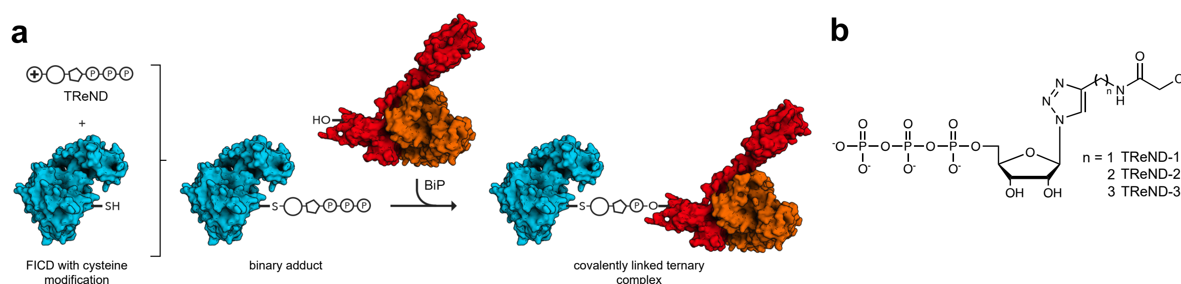

Figure 9 | Principle of the ternary complex formation. a) *Schematic representation of the formation of a covalently linked complex of FICD and BiP using cosubstrate-analogs.* ***b)*** *Chemical structure of the used cosubstrate analogs with varying linker length. TReND means thiol-reactive nucleotide derivative. This figure was taken from Fauser et al 2021.[85] (CC BY 4.0).*

4.2 Identification of targets of AMPylating Fic-enzymes by co-substrate-mediated covalent capture

The article Identification of targets of AMPylating Fic-enzymes by co-substrate-mediated covalent capture *was published in Nature Chemistry in July 2020 (DOI: 10.1038/s41557-020-0484-6). The author of this thesis, Joel Fauser, performed FICD/HYPE-related work regarding molecular biology, protein expression, protein purification, binary adduct formation, pull-downs, and target validation and participated in manuscript writing.*

Cellular processes are often regulated by posttranslational modifications. In this context, the transfer of AMP to host proteins by e.g. the large family of Fic-enzymes has emerged in recent years.[29] However, identification of AMPylation substrates and thus investigation of the physiological role of AMPylation remains challenging due to the lack of generic methods for enrichment of AMPylated proteins.[4] While AMP-specific antibodies, propargyl-equipped ATP

analogs, and isotope-labeled ATP analogs were developed, their application in target identification is limited. While antibodies suffer from bias towards the epitopes they were raised against, ATP analogs compete against high concentrations of cellular ATP and complex mass spectrometry data, respectively.[147,152,153,155,156]

The presented work establishes a novel concept that is based on the production and use of reactive co-substrate-linked enzymes for proteome profiling. Specifically, recombinantly produced enzymes are engineered with a cysteine substitution and are linked to thiol-reactive nucleotide derivatives. This binary adduct is considered as a probe to capture its substrates in a lysate environment, thus permitting enrichment and target identification via LC-MS/MS without competition against endogenous ATP. The concept is validated by the identification of both known and new targets for three different Fic-enzymes, IbpA from *Histophilus somni*, BepA from *Bartonella henselae,* and human FICD/HYPE. The most prominent hits are verified via *in vitro* AMPylation assays and their AMPylation sites mapped. In this study, my expertise on the human AMP transferase FICD/HYPE served to validate the method with the human representative of Fic-enzymes and to derive a general strategy to rationalize cysteine substitutions within Fic-enzymes for binary adduct formation with TReNDs. Therefore, I designed and produced suitable FICD constructs for pull-down assays with FICD binary adducts. With a collaboration partner for LC-MS/MS experiments (Prof. Hartmut Schlüter, UKE, Germany) I showed that the engineered FICD binary adduct is able to capture its physiological substrate BiP in lysates and to *in vitro* AMPylate other enriched proteins such as the eukaryotic elongation factor 1A2 and uridine 5'-monophosphate synthase. Importantly, this study identifies calcyclin binding protein as a new AMPylation substrate of IbpA. While calcyclin binding protein binds a variety of proteins, the interaction profile of its AMPylated counterpart is globally diminished. In addition, the structure of the Fic-enzyme IbpA covalently linked to its substrate Cdc42 is solved, thus demonstrating the structural integrity of the complex upon comparison with the non-crosslinked complex structure.

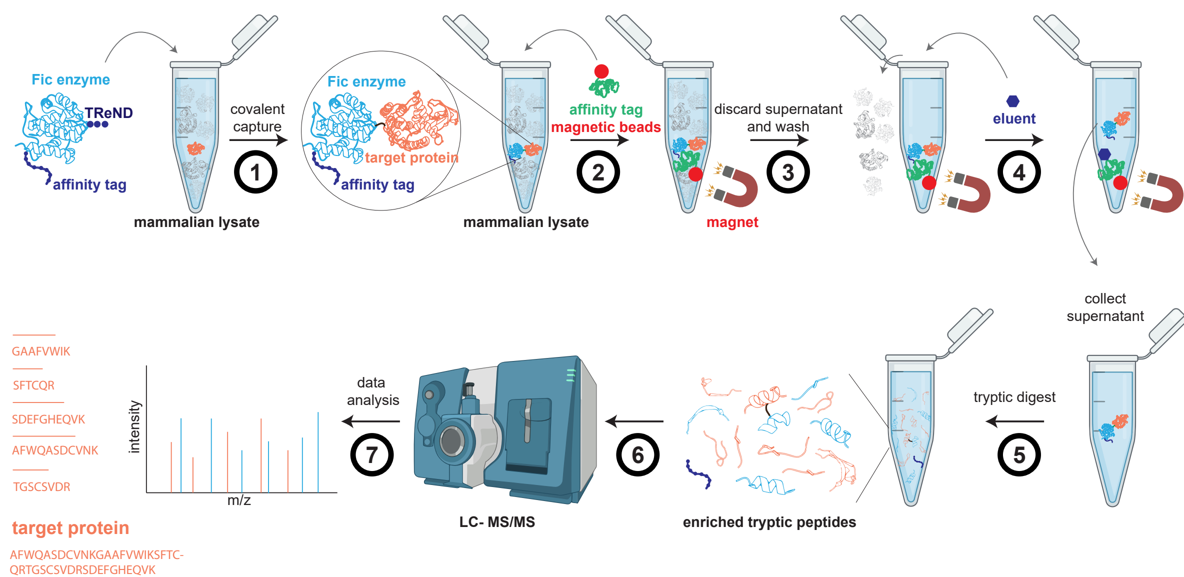

Figure 10 | Principle of target identification by covalent capture. *Fic-enzymes are produced with an affinity tag and engineered with a cysteine substitution for binary adduct formation. Cell lysates are treated with this binary adduct and the protein substrate captured via an AMPylation reaction that yields a covalently linked ternary complex in a lysate environment. The complex is bound to magnetic nanoparticles and enriched via affinity-based methods. The identity of the enriched proteins is determined via tryptic digestion and LC-MS/MS. This figure was adapted with permission from Gulen et al 2020 by Dr. Burak Gülen.*[67]

4.3 Sortase-Mediated Quantifiable Enzyme Immobilization on Magnetic Nanoparticles

The article Sortase-Mediated Quantifiable Enzyme Immobilization on Magnetic Nanoparticles *was published in Bioconjugate Chemistry in July 2020 (DOI: 10.1021/acs.bioconjchem.0c00322). The author of this thesis, Joel Fauser, and Sergey Savitskiy contributed equally to this article. Joel Fauser designed the concept and experiments and wrote the manuscript. Joel Fauser carried out SrtA/IbpA-related work regarding molecular biology, protein expression and purification, peptide coupling, protein immobilization, activity assays, and data analysis.*

Today, systematic identification of enzyme targets strongly relies on mass spectrometry. Methods based on mass spectrometry, however, usually require a preceding process to enrich putative targets via affinity tags. This enrichment procedure often suffers from unspecific binding of proteins to the matrix which increases the complexity of the mass spectrometric data and may even prevent the identification of low abundant protein targets. The envisioned concept would combine (1) thiol-reactive nucleotide analogs that permit covalent capture of AMPylation substrates with engineered Fic-enzymes with (2) site-specific immobilization of the engineered Fic-enzyme to magnetic nanoparticles, ultimately establishing a covalent linkage from the substrate to the matrix. Overall, this work presents preliminary results in method development that would allow covalent, in contrast to affinity-based, enrichment of AMPylation targets.

Initially, a modular platform is introduced that allows enzyme immobilization in two steps. Amine magnetic nanoparticles are functionalized with a short peptide using standard solid-phase peptide chemistry. The N-terminal glycine of the peptide allows subsequent immobilization of Fic-enzymes that carry a C-terminal Sortase A (SrtA) recognition sequence via SrtA mediated protein ligation. The use of GFP-tagged enzymes allows sensitive fluorescence-based quantification of the amount of immobilized enzymes by specific proteolytic cleavage of GFP. To this end, I designed the corresponding peptide and protein constructs and produced both IbpA and SrtA. I performed the functionalization of magnetic nanoparticles with peptides and either performed or supervised the immobilization and quantification of IbpA and GtgE. In kinetic studies of the protease GtgE (conducted by S. Savitskiy) and the Fic-AMPylase IbpA (conducted by me), the functional integrity of immobilized enzymes is demonstrated by their high catalytic activity. In order to exclude catalytic activity mediated by residual soluble enzymes (after enzyme immobilization) I optimized the immobilization and washing procedure and performed corresponding control experiments. Importantly, the immobilized enzymes are also shown to retain high enzymatic activity in a lysate environment. Furthermore, I adapted the concept to show that enzymes can also be immobilized from their N-terminus by functionalization of magnetic nanoparticles with a peptide that carries the SrtA recognition sequence. Finally, the concept is applied for the production of highly pure biological samples intended for Förster resonance energy transfer experiments that were conducted by Vanessa Trauschke.

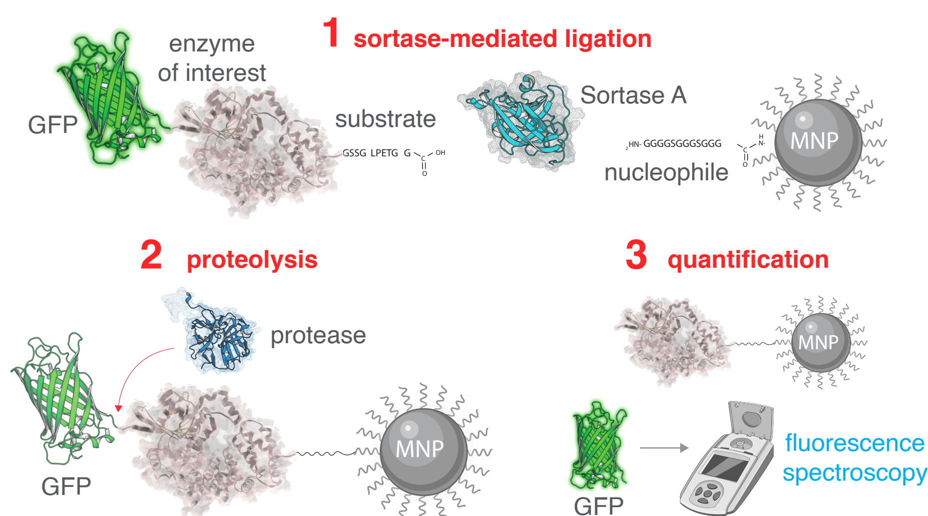

Figure 11 | Schematic summary of Sortase A (SrtA) mediated protein immobilization with subsequent quantification. *Magnetic nanoparticles (MNPs) are equipped with a peptide that bears N-terminal Gly residues. The protein of interest is equipped with an N-terminal GFP-tag and a C-terminal Sortase recognition sequence that allows SrtA mediated protein ligation to MNPs (1). A sequence specific protease is used to cleave the GFP tag that is released from the MNPs (2). Via fluorescence spectroscopy the GFP fluorescence is quantified (3). Reprinted with permission from Fauser and Savitskiy et al, 2020.*[159] *Copyright 2020 American Chemical Society.*

4.4 Current Advances in Covalent Stabilization of Macromolecular Complexes for Structural Biology

The article Current Advances in Covalent Stabilization of Macromolecular Complexes for Structural Biology *was published in Bioconjugate Chemistry in April 2021 (DOI: 10.1021/acs.bioconjchem.1c00118). The author of this thesis, Joel Fauser, conceptualized the article, conducted literature research, and wrote the manuscript.*

The characterization of transient protein-protein and protein-DNA complexes is crucial for the mechanistic understanding of biological processes and advanced applications such as drug development yet poses a great challenge due to the dynamic nature of the interactions. To tackle this challenge, a plethora of concepts were developed to stabilize transient interactions by covalent crosslinking for the structural characterization of macromolecular complexes.

While in the past, mainly unspecific crosslinking methods such as glutaraldehyde crosslinking were employed for stabilization of biological complexes, advances in chemistry now allow researchers to design specific chemical tools to covalently tether enzymes to their substrates enabling subsequent structural analysis via crystallography, cryo-electron microscopy, or nuclear magnetic resonance. Recently, the development of substrate -and co-substrate mediated specific covalent crosslinking opened new avenues in the characterization of otherwise inaccessible transient enzyme-substrate complexes. These unique and innovative concepts now even permit researchers to identify enzymatic mechanisms by capturing specific states of catalysis and there is no doubt that they will continue to be employed for more enzyme classes.

Despite these recent innovations and novel concepts in elucidating protein-protein and protein-nucleic acid complexes via covalent crosslinking, particularly in the last three to five years, the significance of covalent stabilization of macromolecular complexes for structural characterization was not yet reviewed comprehensively. While thematically focused reviews contributed to the use of crosslinking in specific fields, the demand for a general review that assembles both common and novel crosslinking techniques used for stabilization of macromolecular complexes for structural investigation remains unaddressed to date.

The presented review gives a categorical overview of methods that allow specific crosslinking of complexes that proved powerful regarding structure determination. In this context, particular focus is placed on the most recently developed methods such as crosslinking via genetic code expansion, engineered enzyme, or substrate-driven crosslinking, or engineered cosubstrate mediated crosslinking. Furthermore, the review provides future perspectives of crosslinking approaches in structural biology.

5. CONCLUSIONS

Until today, investigation of posttranslational modification faces numerous challenges such as the reliable and sensitive identification of PTM substrates. Moreover, the analysis of the molecular and mechanistic basis for PTM transfer is often hampered by the transient interactions of the modifying enzymes and their dedicated protein substrates. This thesis presents a novel approach to address these challenges by application of synthetic nucleotide derivatives that are demonstrated to covalently link AMP-transferases to their substrates.

Specifically, this approach permitted the structure determination of the previously elusive AMPylation complex of the human AMPylase FICD and its physiological substrate, the Hsp70 chaperone BiP. The resolved complex demonstrates the importance of the TPR motifs of FICD for substrate recognition and uncovers a novel interaction mode of TPR motifs with Hsp70 chaperones. Furthermore, the crystal structure provides the molecular basis for the conformational specificity of FICD.

The presented concept also allows cosubstrate mediated covalent capture of AMPylation substrates by the engineered Fic-enzyme in a lysate environment. The affinity tag of the recombinantly produced Fic-enzyme enables enrichment of covalently linked enzyme-substrate complexes for mass spectrometric analysis. Identification and *in vitro* validation of both known and previously unknown AMPylation substrates of FICD provided the proof of concept. Importantly, the method is superior to more classic pull-down approaches using other cosubstrate derivatives (e.g. N6-propargyl-ATP) since competition with endogenous ATP is bypassed, thus ensuring high sensitivity.

Fic-enzymes themselves can be site-specifically immobilized to a solid support by employing Sortase A mediated ligation. The usage of GFP-fusion constructs allows the sensitive quantification of immobilized enzymes and the confirmation of their functional integrity by activity-based assays. The presented findings provide the basis for the combination of Fic-enzyme immobilization and cosubstrate mediated capture in near future. Covalent pull-downs of AMPylation substrates are expected to improve data quality in mass spectrometry, thus facilitating the detection of low abundant AMPylation substrates.

Overall, the use of cosubstrate derivatives that allow covalent linkage of enzyme-substrate complexes proved powerful for both Fic-enzyme target identification and structure determination of Fic-enzyme-substrate complexes. Its application is anticipated to be exploited not only for other classes of AMP-transferases such as pseudokinases but also adapted to more prominent transferases like kinases.

6. ABBREVIATIONS

Amino acids

Ala/A	alanine	Leu/L	leucine
Arg/R	arginine	Lys/K	lysine
Asn/N	asparagine	Met/M	methionine
Asp/D	aspartic acid	Phe/F	phenylalanine
Cys/C	cysteine	Pro/P	proline
Gln/Q	glutamine	Ser/S	serine
Glu/E	glutamic acid	Thr/T	threonine
Gly/G	glycine	Trp/W	tryptophan
His/H	histidine	Tyr/Y	tyrosine
Ile/I	isoleucine	Val/V	valine

°C	degree Celsius
aa	amino acids
AMP	adenosine-5'-monophosphate
ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
PDB	Protein Data Bank
PTM	posttranslational modifications
min	minute(s)
h	hours
ER	endoplasmic reticulum
α_{inh}	inhibitory α-helix
SOC	super optimal broth with catabolite repression
IPTG	isopropylthiogalactopyranosid
OD	optical density
MBP	maltose-binding protein
GFP	green fluorescent protein
Ni^{2+} NTA IMAC	nickel nitrilotriacetic acid immobilized metal affinity

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8. ACKNOWLEDGEMENTS

First, I would like to thank my supervisor Prof. Aymelt Itzen for the opportunity to do my PhD in his lab, the support both in and outside the lab, the trust in my abilities as well as his constant open door.

I want to thank all collaborators that contributed to this thesis. Prof. Christian Hedberg and Dr. Christian Pett for providing the TReNDs. Prof. Martin Zacharias and Danial Pourjafar-Dehkordi for performing the MD simulations. Prof. Hartmut Schlüter and Dr. Christoph Krisp for conducting the LC-MS/MS experiments.

I am grateful to Dr. Burak Gülen for our friendship, his continuous passion to pass on his expertise in biochemistry and photoshop/illustrator, and his council.

I want to thank Dr. Vivian Pogenberg for his generous support and guidance on biophysical methodologies.

I am grateful to Dr. Sergey Savitskiy, Dr. Max Fottner, and Vanessa Trauschke and for their contribution to the project on Sortase mediated protein ligation.

I want to thank all members of the 'Let's cook' group, foremost Dr. Burak Gülen, Dr. Jiqing Du, and Dr. Sergey Savitskiy for all the joyous evenings we spent together of which many were filled with inspiring discussions and productive working sessions.

I am grateful for all great colleagues in the institute, particularly Dorothea Höpfner, Marietta Kaspers, Gesa König, and Kerstin Schlünz who were always ready to help in many ways.

I want to thank my wife for faithfully supporting me during my challenging PhD journey and being a beautiful mother to our children.

I am grateful to my parents, siblings, and friends for their support and prayers.

I want to thank God for his faithfulness.

9. APPENDICES

Appendix Details	Type
1 Specificity of AMPylation of the human chaperone BiP is mediated by TPR motifs of FICD Fauser J. et. al. Nature Communications doi: 10.1038/s41467-021-22596-0	Publication
2 Identification of targets of AMPylating Fic-enzymes by co-substrate-mediated covalent capture Gulen B. et. al. Nature Chemistry doi: 10.1038/s41557-020-0484-6	Publication
3 Sortase-Mediated Quantifiable Enzyme Immobilization on Magnetic Nanoparticles Fauser J., Savitskiy S. et. al. Bioconjugate Chemistry doi: 10.1021/acs.bioconjchem.0c00322	Publication
4 Current Advances in Covalent Stabilization of Macromolecular Complexes for Structural Biology Fauser J. et. al. Bioconjugate Chemistry doi: 10.1021/acs.bioconjchem.1c00118	Publication

1 Specificity of AMPylation of the human chaperone BiP is mediated by TPR motifs of FICD

Fauser J. et. al., Nature Communications, doi: 10.1038/s41467-021-22596-0

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2 Identification of targets of AMPylating Fic-enzymes by co-substrate-mediated covalent capture

Gulen B. et. al., Nature Chemistry, doi: 10.1038/s41557-020-0484-6

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3 Sortase-Mediated Quantifiable Enzyme Immobilization on Magnetic Nanoparticles

Fauser J. & Savitskiy S. et. al., Bioconjugate Chemistry, doi: 10.1021/acs.bioconjchem.0c00322

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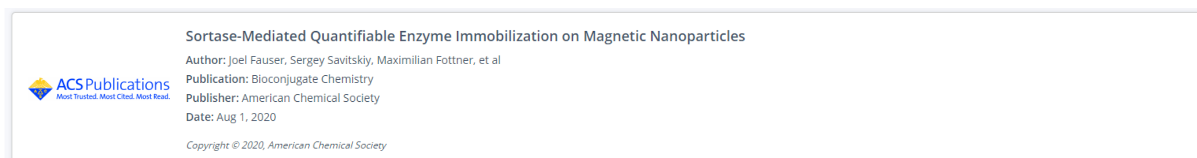

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Author: Joel Fauser, Sergey Savitskiy, Maximilian Fottner, et al
Publication: Bioconjugate Chemistry
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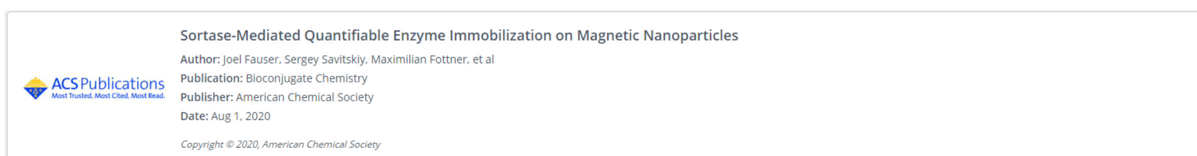

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