PLENARY SPEAKERS

Denise Wootten

Structural and molecular insights into the control of signalling at class B GPCRs

A/Prof Colin Jackson

"Rare" cofactors and pathogens: understanding the role of F420 in Mycobacteria

In this talk, I'll discuss our work on the cofactor F420. Although originally discovered in Archaea, it is present in some bacteria - including Mycobacterium tuberculosis. We have investigated its distribution, physiological roles, and biosynthesis, as well as its role in activating new classes of antibiotic pro-drugs, such as the new anti-TB drugs pretomanid and delamanid. In this context, we have used protein engineering to predict the possible evolutionary routes that M. tuberculosis could take to acquire resistance to these drugs, and using available sequence data have already identified resistant strains in regions where the drugs are yet to be used.

TIR-DOMAIN-ASSEMBLY FORMATION IN TOLL-LIKE RECEPTOR SIGNALING PATHWAYS

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Toll-like receptors (TLRs) detect pathogen- and endogenous danger-associated molecules, initiating innate immune responses that lead to the production of pro-inflammatory cytokines. TLR signal transduction occurs through homotypic interactions between the TLR Toll/interleukin-1 receptor (TIR) domain and TIR domains of the adaptor proteins MAL, MyD88, TRIF, and TRAM. Recruitment of these adaptors via TIR:TIR interactions orchestrates recruitment of downstream effector enzymes, leading to immune responses.

The MAL TIR domain (MAL^{TIR}) has been shown to self-assemble into filaments *in vitro*, form co-filaments with the TLR4 TIR domain ($TLR4^{TIR}$), and induce the formation of MyD88 assemblies. The cryo-EM structure of the MAL^{TIR} filament reveals a proto-filament consisting of two parallel strands of TIR domains in a head-to-tail assembly. To further elucidate the structural basis of TLR signaling and TIR:TIR domain interactions, we sought to determine the structure of $TLR4^{TIR}$:MAL^{TIR} filaments using cryo-EM. Cryo-EM of $TLR4^{TIR}$:MAL^{TIR} filaments revealed two filament morphologies, reconstructions of the two $TLR4^{TIR}$:MAL^{TIR} filaments were resolved to resolutions of 3.8Å and 4.4Å. Modelling of $TLR4^{TIR}$ and MAL^{TIR} subunits within the $TLR4^{TIR}$:MAL^{TIR} filaments indicates MAL^{TIR} forms proto-filaments similar to those observed in the structure of the MAL^{TIR} filament. Furthermore, $TLR4^{TIR}$ subunits form a single strand sandwiched between MAL^{TIR} proto-filaments. The interactions within the $TLR4^{TIR}$:MAL^{TIR} filaments may reflect the biological assemblies and the molecular mechanisms of TIR:TIR interactions between TIR domains in TLR signaling pathways.

A TRANSMEMBRANE ADAPTOR FOR TOLL-LIKE RECEPTORS PROMOTES SELECTIVE PRO-INFLAMMATORY RESPONSES

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Objectives: Toll-like receptors (TLR) activate signalling cascades to control proinflammatory cytokine outputs; adaptor proteins modulate signalling serving to differentiate cytokine programs and regulate inflammation. Transmembrane adaptors (TRAPs) acts as scaffolds for kinases and effectors for signalling from other immune receptors[1], and here we studied the role of a little-known TRAP, SCIMP, in TLR pathways.
Methods: Using GST pull-downs and mass spectrometry, we identified SCIMP as a novel binding partner for TLR4[2]. Site-directed mutagenesis was used to pinpoint key binding residues. Newly developed molecular probes[3] were subsequently used to examine individual TLR-dependent phosphotyrosine sites in SCIMP. SCIMP silencing or knockout and functional studies revealed the role of SCIMP in pro-inflammatory signalling and cytokine outputs.
Results: SCIMP binds directly to the TIR domain of TLR4 via an unusual non-TIR-TIR interaction. Mechanically, SCIMP presents Lyn kinase for TLR phosphorylation and this modulates downstream signalling, resulting in the selective LPS-mediated secretion of the pro-inflammatory cytokines, IL-6 and IL-12p40. SCIMP itself is activated by LPS and other TLR ligands through phosphorylation of individual tyrosines (Y58, Y96, and Y120) that scaffold different effectors, Grb2, Csk, and/or SLP65, with divergent kinetics, varying TLR-mediated outputs.
Conclusions: Our studies implicate SCIMP as a common transmembrane adaptor for TLR tyrosine phosphorylation through its effector proteins and reveal how SCIMP as a proximal adaptor can impart remarkable specificity to a range of TLR-driven inflammatory cytokine responses.

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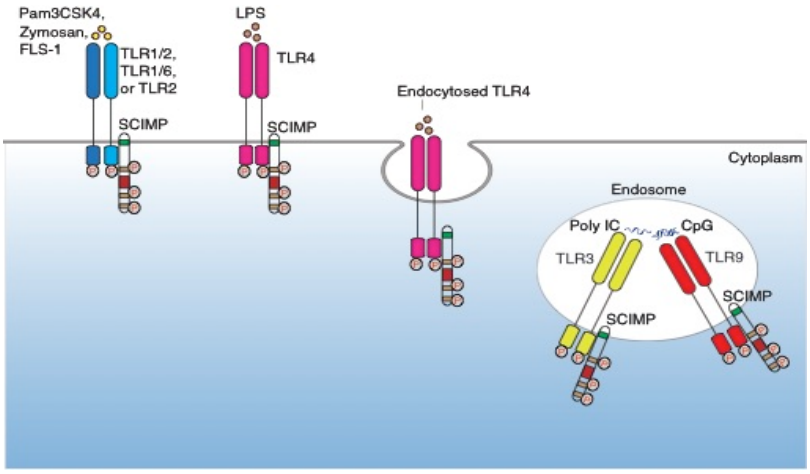

STRUCTURAL AND BIOCHEMICAL CHARECTERIZATION OF THE ENZYMATIC ACTIVITY OF SARM1^{TIR}

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Axon degeneration is one of the starting points for many neurodegenerative diseases including Parkinson's and Alzheimer's disease. According to the World Health Organization (WHO), by 2040, neurodegenerative disease will overtake cancer to become the second leading cause of death after cardiovascular disease. Recently, Sterile alpha- and armadillo-motif-containing protein (SARM1) has been linked to axon degeneration. SARM1 has three domains namely an armadillo repeat domain, followed by two tandem SAM domains, and a C-terminal Toll/interleukin-1 receptor (TIR) domain. Our studies show that the TIR domain of SARM1 has catalytic activity and can cleave NAD^+ and $NADP^+$, which are important co-factors for many biological processes including cellular respiration and maintaining energy at the cellular level. Depletion of NAD+ and NADP+ has been linked to axon degeneration. Furthermore, knockout of SARM1 in mice prevents axon degeneration following axon injury. To date, the key determinants for the regulation and activation of SARM1 are unknown. To characterize the active site and catalytic mechanism of the SARM1 TIR domain we determined the crystallographic structures of SARM1 TIR mutants. In addition, we assessed the role of residues surrounding a previously proposed activity site using site-direct mutagenesis and a fluorescent assay measuring εNAD cleavage. Overall, our results provide a structural basis for the NADase activity of SARM1.

ACTIVATION OF RAB8A BY GUANINE NUCLEOTIDE EXCHANGE FACTORS RABIN8 AND GRAB FOR MACROPHAGE TOLL-LIKE RECEPTOR SIGNALLING.

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The Rab GTPase Rab8a is an important master regulator for many intracellular trafficking and signalling pathways. In macrophages, Rab8a is activated by pathogen signatures such as lipopolysaccharide (LPS) and other Toll-like receptor (TLR) agonists on early macropinosomes, facilitating downstream inflammatory signalling processes. Upon activation, Rab8a directly recruits PI3Kγ as an effector to modulate TLR-induced Akt, mTOR signalling to shape cytokine outputs and inflammation. The guanine nucleotide exchange factors (GEFs) and other proteins responsible for activating Rab8a in this context are not known. To identify candidates, using GST affinity pull-downs and mass spectrometry analysis, we identified the Rab8 specific GEF, GRAB, as a Rab8a binding protein in activated macrophages. GRAB, along with the similarly structured, Rabin8, are two well-known Rab8 GEFs and both were investigated in macrophages. Co-immunoprecipitation and Rab8 nucleotide activation assays showed that both GRAB and Rabin8 undergo LPS-inducible association with Rab8 and are localised on cell surface ruffles and macropinosomes where they coincide with sites of Rab8a signalling. CRISPR-Cas9 mediated knock-out (KO) cell lines of Rabin8, GRAB and double KOs showed that both GEFs contribute to TLR4 induced Rab8a GTP loading, but are not required for its membrane recruitment. In addition, measurement of signalling profiles and live cell imaging of the double KOs revealed that either GEF is individually sufficient to mediate PI3Kγ-dependent Akt/mTOR signalling at macropinosomes during TLR-driven inflammation, suggesting a redundant relationship between these proteins. Thus, both Rabin8 and GRAB are revealed as key positive regulators of Rab8a nucleotide exchange on macropinosomes for TLR signalling and inflammatory programs. These GEFs may be, or may lead to, potential therapeutic targets for manipulating inflammation.

/(Figure 1. Illustration of TLR4-LRP1-Rab8a-PI3Kγ activation pathway in macrophages. Activation of TLR4 by bacterial LPS on dynamic macrophage surface ruffles elicits a signalling cascade through the crosstalk receptor LRP1. This results in Rab8a activation by its GEFs Rabin8 and GRAB, leading to downstream Akt/mTOR signalling that constrains inflammation through cytokine biasing.)

IN VITRO RECONSTITUTION OF CAPSID LATTICE LATTICES TO SCREEN AND STUDY CAPSID BINDERS

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The HIV-1 capsid is made up of ~1500 copies of the viral capsid protein (CA) that assemble into a conical lattice to enclose the viral RNA and associated viral proteins. More than being a proteinaceous shell, HIV-1 uses the CA lattice as an interaction surface to hijack host proteins and small molecules that regulate processes such as cytoplasmic transport, capsid uncoating, nuclear import and integration of proviral DNA. CA mutants with engineered cysteine residues at lattice interfaces self-assemble *in vitro* into a range of cross-linked structures including tubes and cones that resemble to the authentic HIV-1 capsid. Here, we developed two new fluorescence assays utilising these self-assembled CA structures as binding platforms to measure the interactions of host proteins and other macromolecules with capsid.

The first assay utilises total internal reflection fluorescence microscopy (TIRFM) to visualise the dynamic interactions of fluorescent capsid binders with immobilised capsid tubes assembled from CA A14C/E45C. The interaction movies allow us to quantify binding kinetics and stoichiometry of capsid binders interacting with the capsid. The second assay utilises single molecule spectroscopy (SMS)[1] in conjunction with cell-free expression of host cell proteins in a microplate format as a screening technology for the identification of new capsid-binding proteins. Accumulation of GFP-tagged proteins or fluorescent macromolecules on the reconstituted capsid lattice from CA A204C resulted in characteristic intensity spikes in the fluorescence traces allowing us to distinguish binders from non-binders. We demonstrated the application of the assays by measuring competitive interactions at each of the three known binding interfaces on capsid, namely the CypA loop, the CPSF6 binding pocket and the arginine pore at the center of CA hexamers, which binds to nucleotides and recently identified IP6[2].

In combination our assays are suited for medium-throughput discovery of new capsid binders, identification of binding interfaces via binding competition experiments or use of CA mutants and measurement of the mechanistic parameters that govern their interactions with HIV-1 capsid.

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CHD4 SLIDES NUCLEOSOMES BY DECOUPLING ENTRY- AND EXIT-SIDE DNA TRANSLOCATION

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Chromatin remodellers convert the chemical energy of ATP hydrolysis to the mechanical movement of nucleosomal DNA relative to a histone octamer. The molecular basis for this activity is only partially resolved, and it is not clear if the mechanism is conserved among the four classes of remodelling enzyme. Of these enzymes, the least well understood family are the chromo-helicase domain (CHD) enzymes exemplified by CHD4, the remodelling component of the Nucleosome Remodelling and Deacetylase (NuRD) complex. Here, we use single-molecule assays to demonstrate that the flanking DNA enters and exits the nucleosome through two decoupled translocation processes, and the formation of a CHD4-nucleosome complex – even in the absence of nucleotide – is sufficient to prime the system. In combination with recent analyses of yeast remodelling enzymes, our data lead to a model for the mechanism of nucleosome sliding.

RE-SENSITISING ANTIBIOTIC RESISTANT BACTERIA: USING GENETICS AND SINGLE-CELL FLUORESCENCE MICROSCOPY TO CHARACTERISE THE EFFECTS OF DISTRUPTING DNA REPAIR PATHWAYS IN *E. COLI*

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Antimicrobial resistance is a rapidly growing phenomenon that is a major threat to public health. Specifically, antibiotic resistance amongst bacterial pathogens is a grave concern as it presents the possibility of a 'post antibiotic era' in which minor injuries and infections will once again be lethal. Current initiatives to moderate bacterial resistance are inefficient and there is a need for new strategies to contain and reduce antimicrobial resistance. Recent studies have reported many antibiotics appear to have a common bactericidal mechanism that involves the overproduction of reactive oxygen species eliciting lethal DNA damage. If DNA repair were to be compromised, this may offer a new pathway to treat pathogenic bacterial infections. This project begins to address the knowledge gap surrounding DNA repair in antibiotic-resistant *Escherichia coli* (*E. coli*) using genetic modification and advanced single-cell fluorescence imaging to broadly characterise these molecular pathways. Firstly, the minimum inhibitory concentration of ciprofloxacin across *E. coli* strains containing DNA repair gene deletions, *recA*, *recB*, *recF*, *recO*, *recR and mutT,* were analysed on LB/Agar plates using Ciprofloxacin M.I.C.Evaluator™ strips and in serially diluted liquid cultures. Genes associated with double-strand break repair, *recA* and *recB*, significantly reduced the minimum inhibitory concentration of ciprofloxacin-resistance cells on LB/agar to 1.25 $\pm$ 0.5 $\mu g.ml^{-1}$ and 0.14 $\pm$ 0.4 $\mu g.ml^{-1}$, respectively, when compared to wild-type cells (9.00 $\pm$1.0 $\mu g.ml^{-1}$) Re-sensitisation effects were conserved in serially diluted liquid culture with the removal of double-strand break repair, *via* the deletion of *recB,* having the greatest effect. Double-strand break repair is a key activator of the DNA damage response, a system that upregulates DNA repair mechanisms and is thought to switch these repair mechanisms into a mutagenic state, potentially as a means of speeding up evolution to increase a bacterium's chance of survival. To investigate this further, single-cell fluorescence microscopy was conducted to monitor the initiation of the DNA damage response via a reporter plasmid, pUA66 (*sulA-gfp)*. The deletion of *recB* resulted in a decrease in mean cell brightness for both ciprofloxacin-sensitive and ciprofloxacin-resistant strains. The data in this study suggests the deletion of *recB* can effectively re-sensitise ciprofloxacin-resistant *E. coli* and may subsequently remove the DNA damage response and therefore, mutagenic repair conferring resistant alleles. The re-sensitisation effect of deleting *recB* should be considered for other drug-resistant strains to evaluate if re-sensitisation to numerous antibiotics is possible. Furthermore, if the deletion of *recB* can prevent the increase of further resistant alleles it would be a highly favourable target for the development of pharmacological inhibitors. These inhibitors could be used in combination with ciprofloxacin and potentially other antibiotics to treat antibiotic-resistant pathogens.

RESOLVING STALLED REPLICATION FORKS IN *ESCHERICHIA COLI* AT SITES OF NUCLEOPROTEIN COMPLEXES

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DNA replication occurs on template DNA coated in DNA-binding proteins. The inability of the multiprotein replication machine (replisome) to overcome nucleoprotein obstacles has been determined as a major source of genetic instability in many organisms. The bacterial model organism, *Escherichia coli* (*E. coli*), contains proteins, Rep and UvrD, that can remove these nucleoprotein barriers ahead of the replication fork, preventing the replisome from stalling at these sites. The interactions between these proteins and the replisome that contribute to this activity remain unknown. To investigate if Rep and UvrD can rescue stalled replication forks *in vitro* we developed a model nucleoprotein complex using the nuclease dead CRISPR/Cas9 (dCas9) protein. Using bulk biochemical replication assays and single-molecule total internal reflection fluorescence (smTIRF), we show that replication is site-specifically stalled by high-stability dCas9 complexes, evident by the absence of extended DNA products. Further, these roadblocks are displaced by Rep and UvrD, allowing replication to continue, which is indicated by the extension of previously blocked DNA products. These observations provide evidence that Rep and UvrD can remove high-stability nucleoprotein roadblocks preventing replication elongation. Additionally, use of the dCas9 roadblock provides insight into how the replisome may overcome commonly encountered nucleoprotein complexes *in vivo*, for example, RNA polymerase. Further investigations employing fluorescently labelled Rep and UvrD proteins in Single-molecule fluorescence assays will provide further insight into the interactions that contribute to the restoration of replication.

TOWARDS A MOLECULAR UNDERSTANDING OF RETROMER AND SNX-BAR INTERACTION THROUGH THE NOVEL MACROCYCLIC PEPTIDES

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Maintenance of appropriate levels of endocytic trafficking and subsequent sorting in endosomes is essential for every aspect of cellular life. The retromer complex composed of Vps35, Vps29 and Vps26 is an endosome-membrane-associated master conductor for recycling of many cargo transmembrane proteins from endosomes, both to the *trans*-Golgi network (TGN) and the plasma membrane. This trimeric assembly is further formed sub-complexes with numerous accessory proteins, in particular, sorting nexin (SNX) – bin/amphiphysin/rvs (BAR) family members. The association between retromer complex and the SNX-BAR family members Vps5 and Vps17 originally discovered in yeast is known to form a stable pentameric complex. In higher eukaryotes however, the SNX-BARs are more transiently associated with the core retromer complex. Despite the importance of retromer – SNX-BAR complex in endosomal cargo sorting, the molecular basis of how yeast retromer complex forms much stronger interaction with SNX-BARs than its human counterparts remaining poorly characterized.

Recently, we have applied random nonstandard peptides integrated discovery (RaPID) approach to identify a group of retromer modulating cyclic peptides. Through the biophysical and structural analysis of these cyclic peptides, we have identified a novel Vps29 binding motif that also exists in yeast Vps5 but not its SNX1/2 orthologs in higher eukaryotes. Mutation of the key residues significantly reduces Vps5 from binding to the retromer complex. With this novel finding as the basis, we are now processing towards the characterisation of the human retromer complex incorporating SNX1 and SNX5.

DEVELOPMENT OF NA_V-SELECTIVE AGONISTS WITH POTENTIAL FOR TREATMENT OF DRAVET SYNDROME EPILEPSY

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Dravet syndrome (DS) is a catastrophic epileptic encephalopathy characterised by childhood-onset polymorphic seizures, multiple neuropsychiatric comorbidities, and increased risk of sudden death. Most DS cases result from *de novo* loss-of-function mutations in one allele of the *SCN1A* gene that encodes the ion-conducting α- subunit of the voltage-gated sodium channel 1.1 ($Na_V1.1$). In the brain, $Na_V1.1$ is primarily found in the axon initial segment of fast-spiking GABAergic inhibitory interneurons; selective deletion of $Na_V1.1$ in these interneurons in mice recapitulates the symptoms of DS. Thus, the principle mechanism proposed to underlie seizure genesis in DS is loss of inhibitory input due to dysfunctional firing of GABAergic interneurons.

We hypothesised that DS symptoms could be ameliorated by a drug that activated the reduced population of functional $Na_V1.1$ channels in DS interneurons. Toward this end, we identified and characterised two homologous disulfide-rich spider- venom peptides (Hm1a and Hm1b) that selectively potentiate $Na_V1.1$. Hm1a rescues DS interneurons from action potential collapse, while i.c.v. infusion of Hm1a abolishes seizures in DS mice and rescues them from premature death. However, Hm1a is unstable *in vivo* and difficult to produce via both recombinant and chemical methods. We therefore produced recombinant Hm1b using periplasmic *E. coli* expression system and examined its selectivity against a panel of human Na_V subtypes using whole-cell patch-clamp recordings. Hm1b is a potent and highly selective agonist of $Na_V1.1$ (EC_{50} ~12 nM). Hm1b is a gating modifier that shifts the voltage dependence of channel activation and inactivation to hyperpolarised and depolarised potentials respectively, presumably by interacting with the channel's voltage-sensor domains. It has minimal effect on peak current amplitude but robustly inhibits fast inactivation to cause a large increase in sustained currents. Hm1b has no effect on other Nav subtypes except $Na_V1.3$. Like Hm1a, the structure of Hm1b determined using NMR revealed a classical inhibitor cystine knot motif in which the peptide is cross-braced by three disulfide bonds. However, we show that Hm1b is an order of magnitude more stable than Hm1a in human cerebrospinal fluid. Taken together, our data suggest that Hm1b is an exciting lead for a precision therapeutic targeted against DS.

The branched chain amino acid biosynthesis pathway as a route to the discovery of new antimicrobial drugs.

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It is well established that new drugs are needed to combat the threat of the rise in antibiotic resistance. The enzymes that belong to the branched chain amino acid pathway (BCAA) are attractive antimicrobial drug targets. This is because this pathway is not present in humans, whilst the activity of this pathway appears to be essential to the survival of many human microbial pathogens. A comprehensive evaluation of several already well-established inhibitors of the second enzyme in the BCAA pathway, ketol-acid reductoisomerase (KARI), has previously been completed[1-3]. Cyclopropane-1,1-dicarboxylate (CPD), 2-(dimethylphosphoryl)-2-hydroxyacetic acid (hoe 704) and N-isopropyloxalyl hydroxamate (IpOHA) shown to bind competitively and in multi-dentate manner to *S. aureus* KARI with K_i values of 2.73 μM, 0.14 μM and 7.9 nM, respectively[4]. Based on these results, new IpOHA analogs were designed and synthesized. The most potent of these compounds has a K_i value of x nM. Crystal structures of these inhibitors in complex with *S.aureus* KARI have also been determined. In addition to these substrate analogs, the medicines for malaria pathogen box library was screened to try to identify new KARI inhibitors. From this library, an intriguing new compound with a K_i of 531 nM emerged. The crystal structure of this compound in complex with *S. aureus* KARI was determined, showing that a cleaved product was bound in the active site. The analogs of IpOHA were then tested in cell-based studies and shown to have MICs in 6 – 30 μM range for M. tuberculosis, while IpOHA was shown to have an MIC value of 0.32 μM against uropathogenic *E.coli*. These structural insights and cell susceptibility studies will guide new strategies to design new compounds that have increased potency for the enzyme and in antimicrobial assays.

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TARGETING TIR DOMAIN ASSEMBLIES IN TLR SIGNALLING PATHWAYS TO DESIGN ANTI-INFLAMMATORY COMPOUNDS

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Toll-like receptors (TLRs) are central components of host innate immunity. Upon activation, their cytosolic TIR (Toll/interleukin-1 receptor) domains recruit the TIR-domain containing adaptor proteins MyD88 and MAL via TIR: TIR interactions, which triggers downstream activation of the transcription factor NF-κB to induce anti-pathogen responses. MAL works as a bridging adaptor for the association of MyD88 with TLR2 and 4.

Excessive or prolonged activation of this signalosome may lead to chronic inflammatory diseases, so a broad spectrum anti-inflammatory drug can be designed by targeting the TLR2/4 pathways. TLR-antagonist designed against the extracellular domains of the receptors has had limited outcomes. Targeting protein-protein interactions of the downstream adaptors provides an alternative approach.

We have recently shown that the MAL^{TIR} spontaneously and reversibly forms filaments *in vitro* and cryo-EM, mutational and cell-based studies have identified a conserved open-ended mode of TIR domain interaction that is important for the formation of a functional TLR4 signalosomes and NF-κB activation. To identify small molecules that can inhibit the formation of these TIR domain assemblies, a library of 400 fluorinated fragments was screened by ^{19}F-NMR spectroscopy, and 17 hits were identified that bind with MAL^{TIR}. Hits were further validated by HSQC and SPR. SAR by catalogue and molecular docking have been adopted to identify commercial compounds that carry the scaffolds of these hits. Moreover, a number of small molecules have been identified that inhibit MAL^{TIR} assembly *in vitro* at μM concentrations. Analogue exploration, cell-based functional assays, crystallographic and NMR-based studies are underway to define structure-activity relationships of the identified compounds.

INHIBITION OF PROTEIN-PROTEIN INTERACTIONS USING A NEW GUIDELINE FOR PEPTIDE DESIGN

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Many protein-protein interactions are hotly pursued as therapeutic targets. To design drug leads, many academic and pharmaceutical groups have looked to using peptides because they fill the gap between antibodies and small molecules. We have focused on a design approach called grafting, which involves insertion of a bioactive epitope into a stable scaffold, in order to generate a constrained peptide with improved thermodynamic and metabolic properties. However, the lack of systematic approaches for grafting have often resulted in grafted peptides with inactive conformations. Here, by investigating the structure similarity of epitope and scaffold, we find the position of the terminal residues to be a key determinant of conformation for grafting. We propose a guideline for grafting based on structural comparison of peptides with scaffolds, and we show that this novel approach can facilitate the design of stable and active ligands able to inhibit protein-protein interactions.

Reference: Yin, H.; Craik, D. J.; Wang, C. K. Angewandte Chemie International Edition 2019, 58, 7652-7656.

STRUCTURAL CHARACTERISATION OF AN ANTAR DOMAIN ANTI-TERMINATOR PROTEIN BOUND TO RNA

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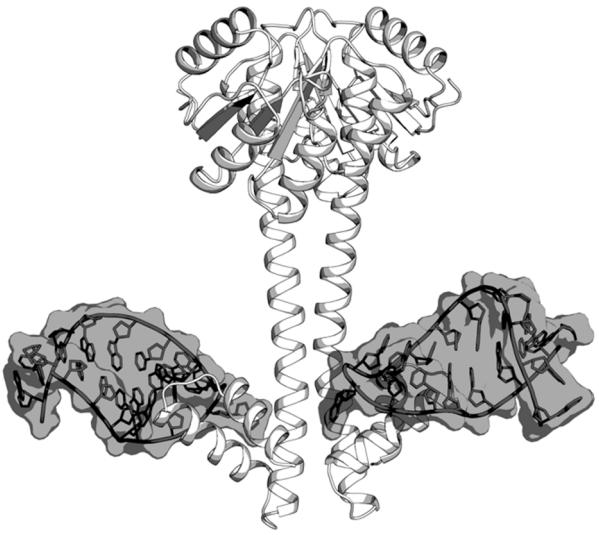
Regulated transcription termination provides an efficient and responsive means to control gene expression. In bacteria, rho-independent termination occurs through the formation of an intrinsic RNA stem-loop (T-loop), which disrupts the RNA polymerase elongation complex, resulting in its dissociation from the DNA template. Bacteria have a number of strategies for over-riding intrinsic T-loops, one of which is the formation of mutually exclusive RNA structures. Proteins that promote or stabilise the formation of these alternative structures are known as anti-terminators.

The ANTAR domains are a family of recently discovered RNA binding anti-terminators that are most commonly associated with two component signalling (TCS) pathways. Unlike the canonical DNA binding output domains of TCS proteins, ANTAR domains facilitate their response by binding to RNA. EutV from *E. faecalis* is an ANTAR domain protein that controls gene expression of the *eut* operon and a model protein for studying ANTAR induced anti-termination. The ANTAR domain binding motif consists of dual RNA hexaloops, with the second hexaloop overlapping with the 5' end of the intrinsic T-loop. Despite the well-defined binding motif, it is unknown at the molecular level how the ANTAR domains interact with RNA.

In this work we have determined the 2.16 Å resolution crystal structure of the ANTAR domain protein EutV in its RNA-free form. The dimeric structure of EutV shows a high degree of similarity to the previously characterised AmiR, suggesting a common dimeric organisation for these domains.

We went on to solve the structure of EutV bound to RNA, which represents the first reported structure of an ANTAR domain bound to its cognate RNA binding motif (see Figure). Our studies highlight the key interactions between conserved EutV residues and the RNA, as well as protein conformational changes undergone upon RNA binding. Furthermore, the structure identified the orientation of the RNA hairpins relative to the ANTAR domains, which invalided the currently accepted model that a EutV dimer can bind to both hairpins simultaneously. These interactions were confirmed by alanine mutagenesis, allowing us to propose an updated model for ANTAR domain anti-termination. One in which a EutV dimer utilises only a single hexaloop of the dual hairpin motif at a time.



STRUCTURAL CHARACTERISATION OF NOVEL TRANSCRIPTION REGULATOR ACAB FOR INCC CONJUGATION

S. J. Hancock[1,2], M. Phan[1,2], Z. Luo[1,2,3], A. W. Lo[1,2], K. M. Peters[1,2], B. M. Forde[1,2], J. Whitfield[3,4], J. Yang[5], R. A. Strugnell[5], D. L. Paterson[6], T. R. Walsh[7], B. Kobe[1,2,3], S. A. Beatson[1,2], M. A. Schembri[1,2]

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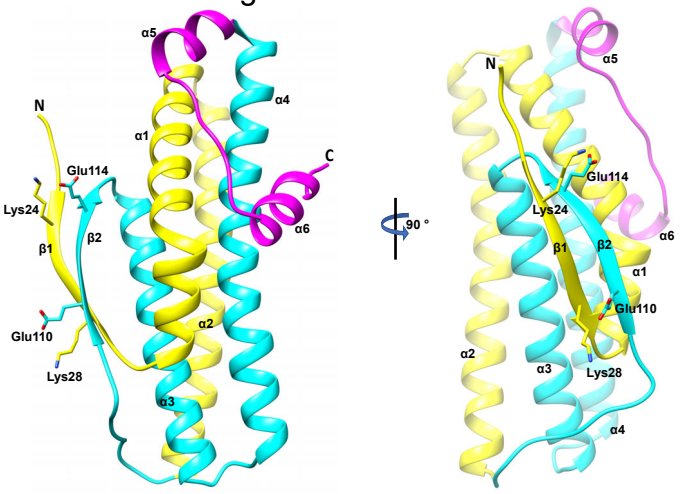
IncC plasmids are clinically important contributors to the spread of antibiotic resistance genes in enteric human pathogens. Especially, these plasmids are associated with the emergence of carbapenem-resistant *Enterobacteriaceae*, owing to their carriage of genes encoding metallo-β-lactamases with carbapenemase activity. One means of plasmids acquisition among bacterial cells is through conjugation. To date, the genetic and molecular details of IncC-conjugation are not clear. In this work, we identified a novel gene, *acaB*, on the IncC plasmid from uropathogenic *E. coli*. We show that this gene encodes a novel transcription regulator, AcaB, that activates conjugation via upregulating the transcription of genes involved in the production of conjugative machinery. Using *in situ* proteolysis and Se-SAD phasing, we determined the crystal structure of AcaB at 2.9 Å resolution. The AcaB structure reveals a novel protein fold that has a distant resemblance to the ribbon-helix-helix superfamily DNA-binding proteins. The protein possesses 2 β-α-α units in tandem, folding into a 4-helix bundle and an exposed 2-stranded β-sheet. Structure-guided functional analyses reveal AcaB binding to DNA occurs via the β-sheet region. This work provides the genetic and molecular basis of IncC conjugation and paves the way for developing new strategies in tackling bacterial multidrug resistance.



BIOCHEMICAL AND STRUCTURAL CHARACTERISATION OF THE COPPER CHAPERONE ACCA FROM NEISSERIA

Denis Thaqi[1], Zhenyao Luo[1], Karrera Djoko[2], Bostjan Kobe[1], Alistair McEwan[1]

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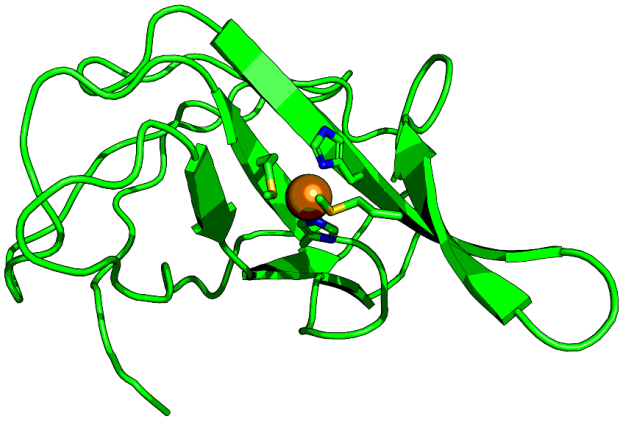
The Neisseria genus contains the obligate human pathogen N. gonorrhoeae, the causative agent of the sexually transmitted disease gonorrhoea[1]. During infection, it undergoes anaerobic respiration via a partial denitrification pathway which utilises the Cu containing nitrite reductase AniA[2]. Cu loading of this enzyme was found to be facilitated by the suspected Cu chaperone AccA, which was identified in Jen et al 2015.

Two suspected Cu binding sites were identified in AccA, and WT and a C terminal deletion mutant were created and expressed to investigate both sites and the first site respectively. Electron paramagnetic resonance (EPR) experiments were conducted on both forms of the protein to examine the Cu binding stoichiometry and information about the Cu binding sites. Continuous wave EPR has identified a roughly 1:1 ratio of Cu(II):AccA for the deletion, and a 1.64:1 ratio for wild type, indicating a second binding site with lower affinity. Pulsed EPR experiments were conducted on the WT protein, indicating Cu binding with His residues, and an approximate distance of 2.6 nm between Cu sites.

Spare matrix screening for WT AccA provided crystal hits only for the copper loaded form, and the following hits were subject to optimisation via changes in solution concentrations and pH. Crystals were diffracted on the MX2 line at the Australian Synchrotron and phased using anomalous scattering from the Cu ions bound to the protein. An incomplete model for WT AccA was created with a resolution of 2.89 angstrom. The model has identified the first Cu site, and identified the binding residues as H96, M80, H103, and M105.

References:
1. Virji, M. Nat Rev Microbiol 2009, 7, 274-286
2. Jen, F.E.-C., Djoko, K.Y., Bent, S.J., Day, C.J., McEwan, A.G., Jenkins, M.P. FASEB 2015, 3828-3838.



STRUCTURAL AND FUNCTIONAL CHARACTERISATION OF THE COMMANDER TRAFFICKING COMPLEX AND ACCESSORY PROTEINS

Michael D Healy 1[1], Ryan J Hall[1], Kai-en Chen[1], Joanna Sacharz[2], Calum J McConville[2], David A Stroud[2], Rajesh Ghai[1,3], Brett M Collins[1]

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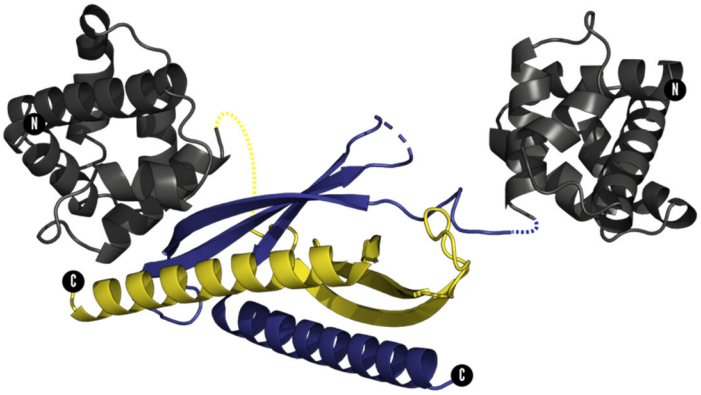
The COMMD proteins are a conserved family of proteins with central roles in intracellular membrane trafficking and transcription. A hallmark feature of the COMMD protein family is a highly conserved C-terminal region, known as the COMM domain. The N-terminal domain of these proteins is highly variable and is proposed to ascribe unique functions to each of the 10 family members. In addition, it is known that COMMDs form oligomeric complexes with each other and act as components of a larger assembly called the Commander complex, which is localized to endosomal compartments and mediates the transport transmembrane cargos including ion transporters and lipoprotein receptors. How these complexes are formed however is completely unknown.

Here we have determined a model of the full length Commd9 (Figure) using X-ray crystallography and X-ray scattering techniques. Our structural analysis revealed that, COMMD proteins possess an α-helical N-terminal domain. While the highly conserved C-terminal COMM domain has a novel structure that is composed of two cone shaped chains that are tightly intertwined with each other to form a globular dimeric module.



In addition, to the structural analysis of individual COMMD family members I have also generated a single novel *E. coli* expression vector that allows for the simultaneous expression of all 10 members of the COMMD family under one promoter. After optimisation of the purification protocol I have been able to isolate 2 distinct subcomplexes, both of which display a propensity to crystallise. Furthermore, initial analysis of CRISPR knockout cell lines via systems mass spectrometry suggest that these sub complexes are not an artifact of the *E. coli* system but are also apparent in mammalian cell cultures. Altogether this work represents a novel way of creating native environments within an *E. coli* expression system, allowing for the reconstitution of entire mammalian complexes.

SOLVING THE STRUCTURE OF YENTC: A STORY OF HYBRID STRUCTURAL BIOLOGY TECHNIQUES

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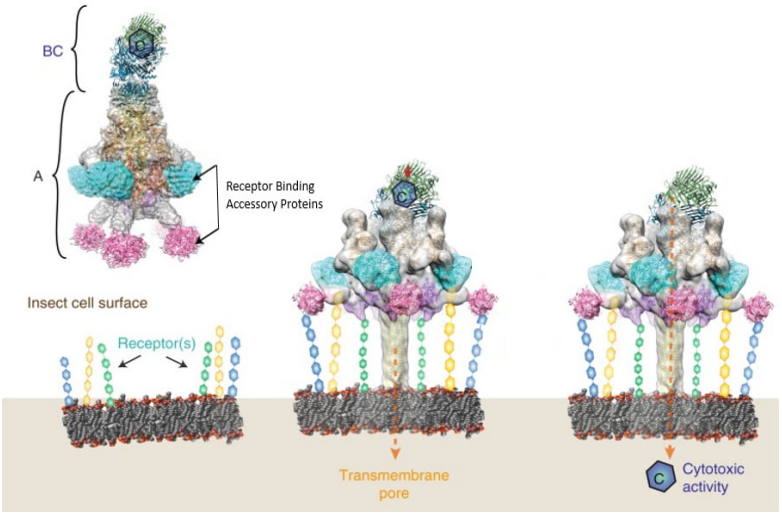
ABC toxin complexes (Tc) are large macromolecular pore forming toxins. They are found in many gram-negative bacteria and are the product of a minimum of three genes; TcA, TcB, and TcC. Tcs are major determinants of toxicity in insect pathogens and are also found in the genomes of vertebrate (including human) pathogens. In addition to being virulence factors, there is potential to exploit them for the development of novel bio-pesticides and antimicrobials. Employing hybrid cryo-electron microscopy, crystallographic, and atomic modelling techniques, we have determined the first structure of a type II ABC Tc, YenTc, found in the entomopathogenic *Yersinia entomophaga* to near-atomic resolution in its pre-pore state. We were also able to determine the structure of YenTc in its pore state at lower resolution. This work suggests an interesting mode of cell recognition that differs from that of type I Tcs and furthers our understanding of large conformational rearrangements of type II Tcs during pore formation and toxin trans-location. The figure below shows the TcA pore-forming apparatus of YenTc, which forms a homo-pentameric shell surrounding a central channel that spans the length of the Tc and is responsible for host cell recognition. TcB and TcC form a cocoon-like structure that encases the cytotoxic carboxy-terminus of the TcC. Upon host cell recognition, large conformational changes take place and plunge the central channel into the host cell membrane in a mechanism synonymous with a hypodermic needle. The toxin TcC and translocated through the Tc and into the host cell where it can exert its effects.



COMBINATORIAL PEPTIDE LIBRARIES FROM CATERPILLAR VENOMS

Andrew A. Walker[1], Samuel D. Robinson[1], Brett Hamilton[2], Andy Sombke[1], Yanni Chin[1], Zoltan Dekan[1], Paul Alewood[1], Christina Schroeder[1], David Merritt[2], Irina Vetter[1,5], and Glenn. F. King[1]

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Whereas adult butterflies and moths are specialised for dispersal and mating, their larvae—caterpillars—are devoted to eating and growth. This otherwise enviable life history leaves the caterpillar with a problem: how can it defend itself from predators while exposed on the leaves it eats, without natural defenses such as teeth or claws, and unable to flee? To solve this problem, caterpillars have evolved a multitude of biological defenses: irritative hairs, toxins that render them poisonous to eat, adhesive droplets, or spines that inject pain-inducing liquid venoms.

Previous studies on caterpillar venoms have focused on the South American saturniid *Lonomia obliqua*, because their venom is capable of killing humans through haemostatic disruption. However, most caterpillar venoms do not produce either haemostatic disturbances or death, but instead produce intense pain and avoidance responses that deter potential vertebrate and invertebrate predators. In addition, phylogenetic and trait analysis across Lepidoptera strongly suggests venom use has evolved independently at least three times, suggesting other groups of venomous caterpillars are likely to have different venom compositions compared to *L. obliqua*.

I will present data recently collected from venoms of five species of Australian caterpillar in the family Limacodidae (nettle caterpillars; Figure). The venom of each species comprises a combinatorial library of more than 100 individual peptide toxins, with minimal content of proteins >10 kDa or enzymes. Three major groups of peptides are evident, including (1) modified versions of insect neuropeptide hormones whose normal function is neuroendocrine signalling through G-protein coupled receptors (GPCRs); (2) disulfide-rich knottins, a class of peptides found in diverse animal venoms that frequently modulate the activity of neuronal ion channels; and (3) linear amphipathic peptides predicted to take helical structures, similar to venom peptides of hymenopteran insects. I will also discuss our current progress in characterising the structure and function of these peptides, and determining the mechanisms by which they induce pain when injected into vertebrates.

ACCELERATING RESEARCH THROUGH PARALLEL PROTEIN PRODUCTION CAPABILITIES

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Protein Expression Facility, The University of Queensland, Brisbane, Queensland 4072, Australia

High-quality recombinant proteins are sought after for diverse industry and research applications. Many applications demand milligram quantities of pure and homogenous protein preparations, placing pressure on researchers to design an effective production strategy. Key factors to consider include the unique nature of the target protein, choice of expression host, incorporation of fusion tags for enhancing solubility or facilitating purification and the intended end application. The integration of quality control measures is also critical to ensure the purified product is correctly folded and functional. A myriad of vector elements, cell strains and culturing conditions to choose from make identifying the most suitable expression parameters for a given project challenging. Selecting one combination of conditions is unlikely to generate sufficient protein for downstream assays, necessitating further experiments to explore alternatives. This sequential approach to protein production is highly inefficient, creating a bottleneck that limits the potential of research projects. The key to eliminating this bottleneck lies in a platform approach where multiple expression parameters are screened in parallel, enabling rapid identification of optimal conditions for scale-up production. Here we present high throughput capabilities that facilitate parallel optimisation of protein expression in *E. coli*, yeast, baculovirus/insect cells and mammalian cells. Critical factors influencing protein expression are explored, enabling efforts to be focused on strategies that maximise chances of success. This is placed in the context of a holistic production approach, considering the impact of such strategies on subsequent protein purification and characterisation. Finally, case studies are presented to demonstrate how these capabilities can be applied in practice to accelerate research outcomes.

UNDERSTANDING PROTEIN-MEDIATED MEMBRANE FUSION: THE CASE OF EBOLA VIRUS

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A key step in the life cycle of an enveloped virus is the fusion between the viral envelope and the target cell membrane so that the viral genetic material can enter the cytoplasm of the host cell. Viral fusion is driven by specific viral fusion proteins that undergo major conformational changes through interaction with the host membrane. Although this general mechanism is shared by all enveloped viruses (such as Influenza, HIV, Dengue and Ebola), the details about the fusion process have remained speculative and largely based on assumptions. Specifically, fusion has been hypothesised to occur via the concerted action of at least three fusion proteins, each protein being a trimer, but this is yet to be supported by experiment.

In this study, using atomistic and coarse-grained molecular dynamics simulations, we examined the behaviour of the fusogenic domain of a single Ebola virus fusion protein in the presence of model eukaryotic membranes composed of phospholipids only or mixed with cholesterol in an equimolar ratio. It was found that the region interacting with the host membrane during fusion stayed parallel to the membrane surface rather than embedding as has been assumed. Importantly, a conformational change within a single fusion protein initiated hemifusion between the viral and host membranes. This suggests that a single fusion protein may be sufficient to induce viral fusion, in contrast with the model with at least three proteins that has often been proposed.

EVOLUTION ON THE MICROSCOPE: EXAMINING THE ROLE OF TRANSLESION SYNTHESIS IN THE DEVELOPMENT OF CIPROFLOXACIN RESISTANCE

Megan E. Cherry[1,2], Daniel Zalami[3], Antoine van Oijen[1,2], and Andrew Robinson[1,2]

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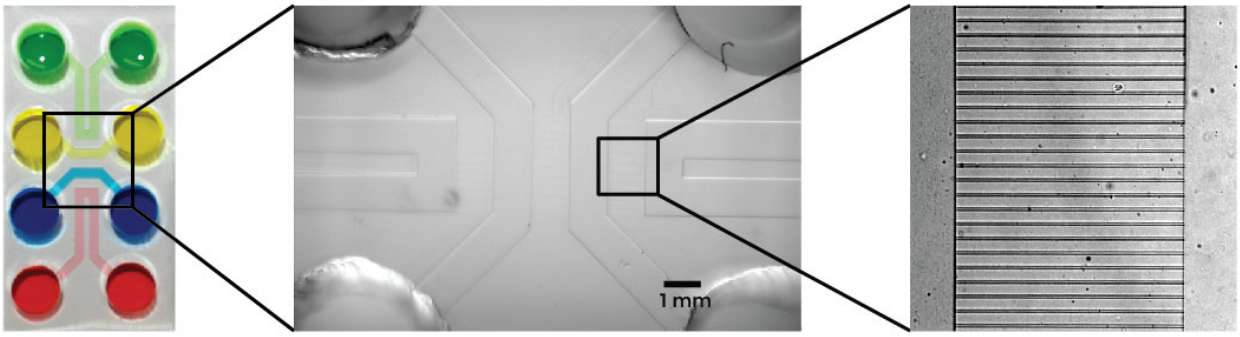
Error-prone translesion synthesis (TLS) is important for the de novo development of antibiotic resistance in bacteria. Unfortunately, little is known regarding the mechanics of this relationship. Recent evidence suggests that mutation supply (population size x mutation rate) is the bottleneck in resistance development and there are, in principle, three main ways in which TLS polymerases could contribute to an increase in mutation supply. First, they could contribute to the genetic diversity present in the bacterial population, prior to selection. Secondly, their activity could promote DNA damage tolerance, thus increasing the size of the population prior to selection. Lastly, these error-prone polymerases could be integral to increasing the frequency of mutations once cells come in contact with antibiotics.

It has previously been impossible to tease out these mechanism subtleties using traditional agar based end-point assays; however, we hypothesize that spatially resolved experimental evolution techniques can be used to distinguish between these three mechanism possibilities. To this end, we have constructed four-channel microfluidic devices in which cells evolve up a continuous gradient of the SOS-inducing antibiotic ciprofloxacin (Figure). By competing TLS-defective and –competent cells, from sub-inhibitory through to inhibitory concentrations, we aim to determine what role each of the TLS polymerases play in the resistance evolution process. To date our observations support an adaptive mutation model by which TLS polymerases promote resistance by increasing the frequency of mutations upon contact with ciprofloxacin.



STALLING, PAUSING, DECOUPLING: SINGLE-MOLECULE VISUALISATION OF A REPLISOME ENCOUNTERING ROADBLOCKS

Stefan H. Mueller[1,2], Lisanne M. Spenkelink [1,2], Jacob S. Lewis[1,2], Grant D. Schauer[3], Harshad Ghodke[1,2], Michael E. O'Donnel[3,4], Antoine M. van Oijen[1,2]

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[4] Howard Hughes Medical Institute.

The process of copying DNA, or DNA replication, is fundamental to all domains of life. DNA replication is carried out by a multi-protein complex referred to as the replisome. Errors occurring during DNA replication can lead to genomic instability and promote diseases such as cancer. Errors can occur due to the presence of roadblocks on DNA like UV-induced lesions, chemically altered bases or DNA-binding proteins. All of these types of roadblocks are universally present throughout the genome. While DNA-repair pathways in cells have been studied extensively, the initial response mechanisms of the replisome to a roadblock remain poorly understood.

We developed an *in vitro* single-molecule assay to visualise individual replisomes encountering a single well-defined roadblock in real time. Using multicolour fluorescence microscopy we obtain spatial and temporal information on single components of a fully reconstituted *S. cerevisiae* replisome encountering roadblocks. We constructed a modular DNA template, containing one of three different roadblocks. First we look at the replisome's response to CPD lesions, the most abundant UV-induced lesion. Second we made a template containing an internal fluorophore. Since this roadblock is directly visible we can determine the fate of the block after bypass or blocking of the replisome. Finally, we use a catalytically inactive CRISPR-Cas9 system, to create a programmable protein roadblock.

We used this assay to elucidate the mechanisms that lead to fork stalling, pausing and decoupling on the single-molecule level. We show that all three types of roadblocks can block replication in our system. Under certain conditions, however, the replisome is inherently capable of bypassing CPD lesions. Furthermore, our results suggest that the essential initiation factor Mcm10 plays a role in the bypass of CPD lesions.

RAPID SELECTION OF STRUCTURALLY DIVERSE AND HIGH AFFINITY CYCLIC PEPTIDE BET BROMODOMAIN INHIBITORS

Karishma Patel[1], Louise J. Walport[2], James L. Walshe[1], Paul Solomon[1], Jason K. K. Low[1], Daniel H. Tran[3], Lorna Wilkinson-White[1], Ana P. G. Silva[1], Jacqueline M. Matthews[1], J. Mitchell Guss[1], Richard J. Payne[3], Hiroaki Suga[4], Toby Passioura[1], Joel P. Mackay[1]

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[3]School of Chemistry, The University of Sydney, Sydney, NSW, Australia.
[4]University of Tokyo, Tokyo, Japan

Macrocyclic peptides have emerged as versatile scaffolds for the design and development of diverse and potent bioactive molecules. Often, these peptides exhibit extraordinary selectivity and high affinity for their targets and their potential to be adapted as therapeutics is reflected by the widespread abundance of functional macrocyclic peptides in nature.

Random nonstandard Peptide Integrated Discovery (RaPID) is a ribosomal synthesis-based technique that has been developed to enable the discovery of effective ligands for any protein of interest. RaPID libraries reach enormous sizes (typically $>10^{12}$) and can also incorporate non-standard amino acids. We have used RaPID to isolate cyclic peptide inhibitors of the acetyllysine-binding bromodomains of the Bromodomain and Extraterminal domain (BET) family of epigenetic regulators. Chemical inhibition of BET proteins shows considerable promise as a therapeutic intervention for a range of diseases, most prominently cancer. Unfortunately, currently available inhibitors exhibit little to no selectivity between BET-family proteins, raising concerns about off-target effects.

Using surface plasmon resonance, we show that the peptides enriched by our RaPID approach boast superior selectivity and affinity to previously developed inhibitors. Crystal structures of several cyclic peptide-bromodomain complexes demonstrate significant structural diversity among the different peptides isolated from the screens. Our findings provide insight into the mechanistic basis behind these high affinity interactions and should allow us to engineer further improvements to turn these peptides into viable drug candidates.

Single-molecule imaging of DNA polymerase II in live *Escherichia coli* cells: housekeeping and DNA damage-induced activities

Thomas J. Armstrong[1,2], Megan E. Cherry[1,2], Elizabeth A. Wood[3], Michael M. Cox[3], Antoine van Oijen[1,2], Sarah S. Henrikus[1,2] and Andrew Robinson[1,2]

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DNA polymerase II (Pol II) is one of three specialised polymerases to be upregulated following DNA damage in *E. coli*. Pol II differs from other damage inducible polymerases (Pols IV and V), in that it belongs to the B family and includes 3′–5′ proof-reading exonuclease activity. Many decades of study have yet to reveal a clear biological role for Pol II. To gain further information into potential biological roles of PolI II, we have turned to live cell single-molecule imaging.

Previously, we have imaged Pols IV and V to reveal novel and unanticipated aspects of their activities. By imaging Pol II in cells, we have now completed the set. We observe that Pol II forms transient foci in the absence of exogenous DNA damage, indicative of short-lived DNA binding activity (~50 ms). This suggests that Pol II has a housekeeping function. Pol II foci were also seen to colocalise much more tightly with replication fork markers and to a higher extent than both Pols IV and V (15–20% colocalise with replisomes).

Significant changes in Pol II behaviour are observed in cells treated with the DNA damaging antibiotic ciprofloxacin. Within minutes of ciprofloxacin treatment, the colocalisation of Pol II with replisomes drops to baseline levels. Over the next 60 minutes colocalisation levels partly recover (~10%). Pol II foci behaviour over this time also changes, with foci becoming much longer lived (~1 s). We are currently investigating whether this change in behaviour stems from the polymerase or exonuclease. The damage-induced drop in colocalisation is lost in RecFOR mutants suggesting that Pol II acts on single-stranded DNA gaps behind the replication fork. Finally, experimental evolution measurements show cells lacking Pol II develop ciprofloxacin resistance faster than wild-type cells indicating a major activity of Pol II is proof reading in ciprofloxacin treated cells.

Enzyme choreography during lesion skipping at the *E. coli* replication fork

Gurleen Kaur [1], Jacob S. Lewis [1,2] , Lisanne M. Spenkelink [1], Slobodan Jergic [1], Andrew Robinson [1,2] and Antoine M. van Oijen [1,2]

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Replisomes are the multi-protein complexes that coordinate the enzymatic activities required for DNA replication. How replisomes bypass lesions on the template DNA during replication has been studied using conventional ensemble biochemical methods. The proposed models of lesion bypass by replisomes in bacteria do not account for the inherit plasticity of the replisome during replication. Where various components may exchange into the replisome in a manner dependent on their concentration. Currently there are no suitable DNA substrates containing site-specific lesions suitable to study *E. coli* DNA replication using single-molecule fluorescence microscopy. To determine the molecular details of replisome bypass of template DNA lesions at the single-molecule level, a linear DNA template containing site specific lesions is required. To date we have designed and constructed a modular linear DNA substrate that is readily visualized with single-molecule resolution. Moreover, using *E. coli* replisomes reconstituted from purified proteins we have observed rates of replication consistent with previous studies. Continued development of this single-molecule assay will allow for investigation of the interplay between leading-strand lesion skipping and polymerase exchange dynamics.

STRUCTURAL BASIS OF SARM1 REGULATION

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Sterile alpha and armadillo-motif containing protein 1 (SARM1) is a well-conserved protein composed of an N-terminal armadillo repeat motif (ARM) domain, two central tandem sterile alpha-motif (SAM) domains and a C-terminal Toll/interleukin-1 receptor (TIR) domain. SARM was first identified as an adaptor protein involved in negative regulation of Toll-like receptor (TLR) signalling. However, recent studies have revealed its role in neuronal development and cell death, which suggests its potential as a therapeutic target.

SARM1 promotes injury-induced axon degeneration, an early and prominent feature of many neurodegenerative diseases, such as Alzheimer's and Parkinson's disease. Truncations of SARM1 show that both the SAM and TIR domains are required for SARM-mediated axon degeneration. The SAM domain is responsible for the oligomerisation of SARM1 and the TIR domain is the enzymatic domain that functions in NAD^+ cleavage. There is evidence the ARM domain auto-inhibits the neurodegeneration activity of SARM1. The structural basis behind this self-regulation mechanism is unclear.

To understand the structural basis of this self-regulatory function, structural information of ARM domain is required. We have successfully expressed, purified and crystallised the ARM domain of Drosophila SARM1. Despite a lot of effort, only needle crystals could be obtained that showed poor diffraction. Recently, we managed to get three dimensional crystals by co-crystallising the protein with nicotinamide mononucleotide (NMN), a nucleotide precursor of NAD^+. Currently, we are using different approaches to solve the phase problem in order to determine the structure.

We also have expressed a protein construct comprising the ARM-SAM domains of human SARM1 ($hSARM1^{ARM\text{-}SAM}$). Negative-stain electron microscopy revealed ring-shaped structures of varying sizes. The ring-shaped structures observed are consistent with the recently solved crystal structure of the SAM domains from human SARM1 (Horsefield *et al*, unpublished), which suggests that the ARM domain does not inhibit the activity of SARM1 by disrupting SAM domain-regulated oligomerisation.

Poster presentations
(Day 2)

1

BRIGHTER RED FLUORESCENT PROTEINS DISPLAY REDUCED STRUCTURAL DYNAMICS

Adam M. Damry[1], Serena E. Hunt[2], Natalie K. Goto[2], Roberto A. Chica[2]

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Red fluorescent proteins (RFPs) are genetically-encoded fluorophores that are extensively used in biological research. For all imaging applications, brighter variants are desired. Brightness is directly proportional to quantum yield (QY), and QY improvements can theoretically be achieved by decreasing dynamics of the chromophore responsible for fluorescence by optimizing packing interactions. Although it has been demonstrated that optimization of local packing interactions around the chromophore can provide brighter FPs, mutations proximal to the chromophore often cause unwanted hypsochromic shifts in emission wavelength. Distal sites provide the possibility of rigidifying the chromophore through trickle-down dynamics without directly affecting its electrostatic environment, but the magnitude and extent of their contributions to dynamics has never been systematically evaluated.

Here, we study this relationship using nuclear magnetic resonance (NMR) spectroscopy of a family of related monomeric RFPs with a range of QY between 0.02 and 0.70. A residue-by-residue comparison using $^{1}H–^{15}N$ HSQC spectra showed line-width broadening correlating with QY in roughly 12% of backbone amide peaks. As peak line-widths are influenced by microsecond–second timescale motions, T1 and T2 relaxation measurements were performed to probe picosecond–nanosecond timescale dynamics. These measurements showed that apparent correlation time increases with QY in roughly 6% of backbone amide peaks. While many positions selected by these experiments are dispersed throughout the RFP scaffold, the β strand 7–10 region shows a cluster of residues whose dynamics correlate with QY. This indicates a potential link between dynamics in this region and chromophore flexibility and brightness. To further probe this relationship, we saturated six sites along this face in mPlum-E16P (QY = 0.13), resulting in the production of mutants displaying QY enhanced by up to 35% without significant alteration of their emission wavelength. Our results show that RFP QY can be increased through mutagenesis of sites distal to the chromophore identified by NMR, opening the door to the rational design of more rigid, brighter RFPs.

2

PEPTIDE-BASED DRUGS TO INHIBIT LDHA, A POTENTIAL TARGET FOR CANCER THERAPY

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Lactate dehydrogenase A (LDHA) is overexpressed in many tumours, particularly in those with high metastatic potential. Previous studies using chemical inhibition and RNA silencing showed that LDHA is involved in tumour initiation, maintenance and proliferation by promoting glycolysis (1). People carrying LDHA deficiency do not show a severe phenotype and can have a healthy live. Therefore, LDHA is a very attractive target for anticancer therapy (2). Great effort has been done to identify small-molecule drugs that inhibit LDHA by competing with the substrate for its active site, but this strategy has not identified molecules able to target LDHA efficiently inside cells (3).
In this project, we use a different approach based on peptides, an attractive alternative to small-molecules to target protein-protein interactions (PPIs) due to their larger interface that allows higher specificity (4). Since LDHA is only active on its tetrameric form, we developed peptides that inhibit the oligomerisation of the enzyme and consequently, its activity. Our peptides also have to be highly stable against proteases and able to reach the cytosol. To achieve that, we use cyclic cell penetrating peptides and combine computer-based predictions, high-throughput screening, and rational design to identify sequences with the ability to target and inhibit LDHA inside cells. This approach is novel and will challenge the landscape of drug discovery programs exclusively dedicated to small-molecules that follow the Lipinski's rule of 5.

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3

DEVELOPING MOLECULES THAT TARGET AN ESSENTIAL BACTERIAL PROTEIN FOR BROAD-SPECTRUM ANTIBIOTICS

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Given the increasing incidence of antibiotic-resistant infections, antibiotics that employ new strategies are urgently needed. Bacterial survival is dependent on proper function of the signal recognition particle (SRP; a protein:RNA complex) and its receptor (FtsY). Deletions of SRP/FtsY components or mutations that inhibit SRP/FtsY interactions have been shown to result in severe bacterial growth defects or cell death (1). A unique set of interactions within the FtsY:SRP-RNA complex represent a novel target that has never been previously pursued for antibiotic development. Furthermore, the nature of the interactions is unique to prokaryotes, minimising any potential off-target effects in humans.

We have previously used a Fragment Based Drug Design approach to identify fragment hits that bind FtsY (2). We are currently using a combined approach involving medicinal chemistry, structure-guided design, molecular docking, biophysical binding experiments and whole-cell activity assays to develop structure-activity relationships (SAR) for the fragment hits and their derivatives. Here, we will report some of our recent work towards developing a set of tool compounds with increasing binding affinity, specificity and biological activity; with the ultimate goal of developing them into antibiotic leads.

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4

VISUALISING HIV CAPSID UNCOATING VIA FLUORESCENCE MICROSCOPY

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Timely disassembly of HIV-1 capsid, the protein shell that houses viral RNA, is essential for successful replication in cells. The disassembly of the capsid shell into its constituent capsid proteins, a process also known as uncoating, is poorly understood. Various biochemical and biophysical uncoating assays have been established to gain insight into the mechanisms governing viral uncoating. Recently, we reported an *in vitro* single molecule fluorescence imaging assay to investigate the uncoating kinetics of viral particles, whereby the precise time of capsid opening is detected by the release of GFP packaged as a solution phase marker inside the capsid (Márquez et al, eLife 2018;7:e34772). The assay requires engineering of proviral plasmids to contain sequences encoding the desired fluorescent tag at a suitable location of the viral genome, which can be cumbersome when working with different viral strains or capsid mutants. Additionally, the encapsidation of GFP inside the viral particle could compromise viral maturation and/or the intrinsic stability of the capsid.

In order to expand the applicability of our uncoating assay to different types of lentiviruses without the need for encapsidation of GFP, we have utilized a range of fluorescently labelled binding proteins (FBP) that can bind to targets inside the viral capsid upon uncoating. As a proof of concept, we immobilised viral particles containing GFP on the coverslip as in our previous approach and then added FBP to the solution. Using total internal reflection fluorescence microscopy, we then followed the fluorescence intensity of GFP and of FBP at the single-particle level. Release of the encapsidated GFP measured at individual viral particles correlated with an increase of the corresponding FBP signal. Our preliminary data suggest that target specific FBP could be generic probes for imaging-based detection of capsid opening that can be adapted to study viral uncoating of various retroviruses and their mutants. The new approach could therefore provide valuable insights into the early retroviral lifecycle. We are currently utilising this approach to unravel the role of small molecules such as IP_6 and nucleotides on the interplay between reverse transcription and HIV-1 capsid uncoating.

5

CRYSTAL STRUCTURE OF A PUTATIVE SUGAR-BINDING PROTEIN FROM MARINE *SYNECHOCOCCUS*. CAN SUBSTRATE-BINDING PROTEINS REDEFINE NUTRIENT ACQUISITION IN MARINE CYANOBACTERIA?

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Substrate-binding proteins (SBPs) are a diverse superfamily of proteins found in prokaryotes, implicated in the uptake of diverse nutrients such as metals, carbohydrates, and amino acids, in conjunction with ABC transporters. Structurally and mechanistically, SBPs are highly similar, with substrate binding known to induce a conformational change, bringing the two component Rossmann domains together to sequester its specific ligand. Having evolved for a wide array of substrates, however, functionally-related SBPs exhibit limited similarity at the sequence level.

From 1229 SBPs in *Synechococcus* ecotypes found in over 300 Australian ocean metagenome samples, 110 were selected for recombinant production and characterisation. I report the X-ray crystal structure (2.8 Å) of an SBP loosely annotated to bind carbohydrates. While having tertiary similarity to carbohydrate- binding SBPs, such as the archetypal maltose-binding protein (MBP), this protein appears to have a requirement for zinc that is previously undocumented.

Marine-dwelling organisms must compete for vital resources in nutrient-limited environments. *Synechococcus* are believed to synthesise their essential nutrients *de novo*, but evidence from the large numbers of SBPs encoded within their genomes suggests otherwise. Questionable sequence annotations therefore occlude ligand specificity, and thus biological function must instead be answered from a structural perspective.

6

CRYSTAL STRUCTURE OF MESO-2,3-BUTANEDIOL DEHYDROGENASE (BDDH) FROM *SERRATIA MACESCENS* H30 IN A COMPLEX WITH NAD+ AND ACETOIN AT 2.0 Å RESOLUTION

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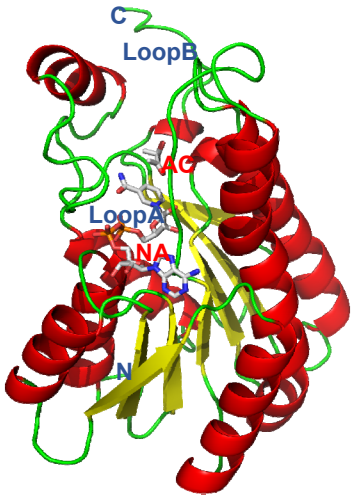
2,3-Butanediol dehydrogenase (BDDH) reversibly catalyzes the conversion of acetoin to 2,3-butanediol. BDDH from the strain *Serratia marcescens* H30 (*sm*BDDH) is a short-chain dehydrogenase/reductase (SDR) and has meso-2,3-BDDH stereospecificity. In this study, *sm*BDDH was expressed and purified to homogeneity. Its specific activity is 239 U/mg for the oxidation reaction and 363 U/mg for the reduction reaction. To understand how stereospecific transformation is achieved by this enzyme, we determined the structure of a complex with its substrate, acetoin, at 2.0 Å. A comparison with *Klebsiella pneumoniae* IAM 1063 (*kp*BDDH) and *Brevibacterium saccharolyticum* C-1012 (*bs*BDDH) shows that *sm*BDDH has the similar overall structure with a central β-sheet flanked by three α-helices on each side (see figure) [1, 2]. However, mobile loops at positions 181-193 (loop A) and 244-251 (loop B) are found to stabilize the cofactor and substrate. In the active site, catalytic residues Ser^{138}, Tyr^{151} and Lys^{155} are conserved in these three enzymes, but the residues (Ser^{140} and Gln$^{247'}$) which is unique in *sm*BDDH may form hydrogen bonds with the hydroxy group at C3 carbon of acetoin.



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7

STRUCTURAL CHARACTERIZATION OF HIGHER-ORDER ASSEMBLY IN TOLL-LIKE RECEPTOR PATHWAYS

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Innate immunity is crucial for preventing organisms from infection by harmful agents. Toll-like receptors (TLRs) play a fundamental role in initiating immune response via recognition of pathogen-associated molecular patterns (PAMPs) in vertebrates, which leads to the production of pro-inflammatory cytokines (Gay & Gangloff, 2007). The Toll/interleukin-1 receptor (TIR) domains of TLRs are responsible for transmitting extracellular signals to intracellular cytoplasmic TIR domain-containing adaptor molecules via TIR: TIR domain interactions (Kawasaki & Kawai *et al.,* 2014). Since it has been previously demonstrated that higher-order assembly formation occurs in the TLR4 signaling pathway, and the mechanism, which is known as signaling by cooperative assembly formation (SCAF), is predicted to occur in all TLR signal transduction. The TLR2 can form heterodimer when binding with different types of ligands. Here we aim to demonstrate the molecular architecture of higher-order assemblies formed by TIR domains with a focus on assemblies in the TLR2-TLR1/6 signaling.

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8

STRUCTURAL CHARACTERIZATION OF ZN-BINDING PROTEINS IN *STREPTOCOCCUS PNEUMONIAE*

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Streptococcus pneumoniae is a critical human bacterial pathogen that causes more than 1 million deaths every year. Zn2+ is one of the essential metal nutrients in all living kingdoms. AdcAII is Zn2+-specific solute-binding protein (SBP) that belongs to the ATP-binding cassette transporters system. Polyhistidine triad protein D (PhtD) belongs to histidine triad protein family, which associates with AdcAII to capture zinc in Streptococcus pneumoniae. To date, the mechanism of PhtD is still unclear, becaused of the lack of the PhtD crystal structure. PhtD contains 5 histidine triad motifs, but there are only two structures of histidine triad motifs available. These two structures indicated that histidine triad motifs have the right geometry for zinc binding. In this work, based on the secondary structural prediction of PhtD, we designed two constructs. The first construct contains the first 3 histidine triad motifs (HT1-3) residues 50-329, whereas the second one contains the last 3 histidine triad motifs (HT3-5) residues 269-587. We attempted to express and purify these two protein constructs for use in crystallization studies. Two rounds of expression and purification were used to obtain high yield and purity proteins. The initial crystals of PhtD HT1-3 and PhtD HT3-5 were obtained from the sparse matrix screening method. However, despite the reproducing efforts, the crystals could not be reproduced. Future work will focus on improving the proteins stability and find the suitable concentration of PhtD HT1-3 and PhtD HT3-5 for crystallization.

9

BRANCHED AMINO ACID BIOSYNTHESIS – AN IMPORTANT ANTITUBERCULOSIS DRUG TARGET

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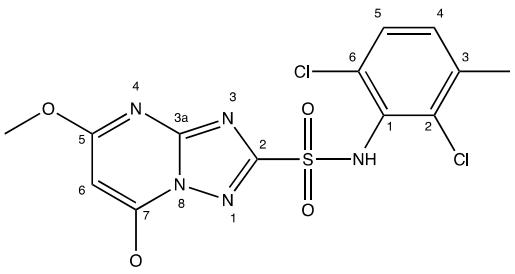
Tuberculosis is a major threat to global human health and is responsible for more than a million deaths each year (1). In addition, the increase in the emergence of multi-drug resistant strains of *Mycobacterium tuberculosis* (*Mtb*) highten the need to discover new drugs with novel modes of action. Enzymes in the BCAA pathway are targets of interest because the activity of the BCAA pathway seems essential in these bacteria for their survival (2,3) but is absent in humans. Thus, compounds that inhibit the BCAA pathway are likely to be non-toxic to humans. In this study, the focus is on acetohydroxyacid synthase (AHAS) which is the first enzyme in branched amino acid pathway. Resazurin microtiter assay (REMA) was used to find out minimal inhibitory concentration (MIC_{90}) of the AHAS inhibitors against *Mtb* strain H37Ra. Three AHAS inhibitors from the triazolopyrimidines family of compounds successfully inhibit the growth of H37Ra with MIC_{90} values <0.5 μM, whilst six other AHAS inhibitors have a moderate effect of MIC_{90} < 20 μM. This study suggests that AHAS inhibitors are good antituberculosis drug candidates.



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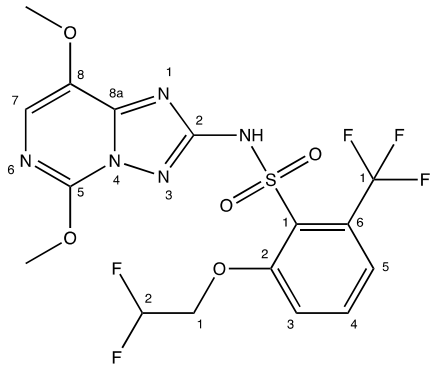

Penoxsulam

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10

COMPOUND LIBRARY SCREENING TO IDENTIFY NOVEL INHIBITORS OF MYCOBACTERIUM TUBERCULOSIS KETOL-ACID REDUCTOISOMERASE

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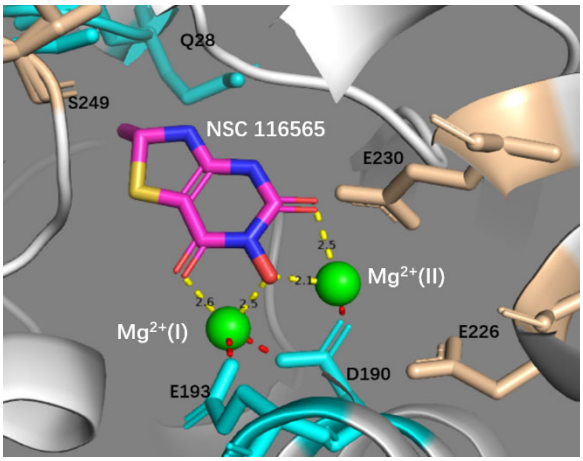
Tuberculosis (TB) is a life-threatening disease that has plagued humanity for thousands of years (1). Even today, it still accounts for more than 1.5 million deaths per annum so remains as a serious problem (2). A possible new target for drug discovery is ketol-acid reductoisomerase (EC 1.1.1.86, KARI), which is the second enzyme in branch chain amino acid (BCAA) biosynthesis pathway. An advantage in targeting this pathway is that it is only found in bacteria, plants and fungi, but not in humans. Thus, the risk of compounds that inhibit this enzyme also being toxic to humans is low.



In all, 2542 compounds from the National Cancer Institute library were screened using a spectrophotometric assay against *Mycobacterium tuberculosis* (*Mt*) KARI. The results showed that 12 compounds in this library inhibited the enzyme by more than 20% at a concentration of 10 μM. Of these, one compound (see Figure), showed strong inhibitory activity with a K_i of 1.8 ± 0.2 μM. To investigate its mode of binding, molecular docking into the crystal structure of *Staphylococcus aureus* (*Sa*) KARI, a homologous enzyme to *Mt* KARI was performed (3) (See Figure). Based on a chemical structure similarity search an enriched compound set is now being developed for further evaluation.

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11

Structural studies of a dodecameric Ketol-acid reductoisomerase.

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Isobutanol, can be synthesized in a cell free cascade using five enzymes and starting from pyruvate as the initial substrate. Three of the enzymes in this pathway are catalytically efficient, but the second enzyme, ketol-acid reductoisomerase (KARI), has a relatively low turnover number and generally requires NADPH (an expensive cofactor) for activity. Here, we are exploring the properties of a KARI isolated from *Campylobacter jejuni* (*Cj* KARI) to determine if it could be a useful enzyme for inclusion into the cascade. To date, we have determined that the protein is dodecameric and, unusually, it can use either NADPH or NADH as a cofactor, NADH with an efficiency 10-fold lower than for NADPH. This enzyme is stable at temperatures as high as 55°C, a property than makes it suited for industrial applications. To further characterize the protein, small angle X-ray scattering (SAXs) has been performed, which confirms in solution that the protein is dodecameric. An initial Cryo-EM 3D model of the structure has been determined, this at 7 Å resolution. The structural data will provide useful information relevant to this enzymes substrate and cofactor specificity, explain why Mg^{2+} is the only metal ion that it can use, and also explain its preference for NADPH as a cofactor. Furthermore, it will be useful as a starting point for rational molecular design approaches aimed at making versions of this enzyme that could be highly suited for industrial applications.

12

THE CHARACTERISATION OF SCIMP AND TOLL-LIKE RECEPTOR COMPLEX

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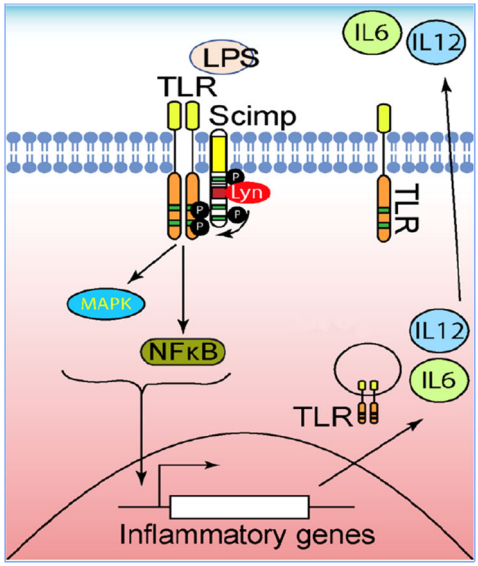
The innate immune system is the first line of defense in response to danger signals from pathogen invasion in all-higher organisms. Toll-like receptors (TLRs) expressed by immune cells, such as macrophages and dendritic cells, are the principal family of molecular sentries for the recognition of conserved microbial structures. Upon activation by various stimuli, TLRs recruit many types of adaptors to trigger proinflammatory signaling cascades, which will further produce inflammatory cytokines that are required for orchestrating wider immune responses.

Classic adaptor proteins include MyD88, MAL, TRAM, TRIF, SARM and BCAP. They all contain TLR domain in their structures. Ligand-bound and dimerised TLRs recruit these adaptors via TIR-TIR interaction in order to initiate MyD88-dependent or TRIF-dependent TLR signaling and further induce specific cytokine production.

Recently, we identified SCIMP as the first non-TIR containing transmembrane adaptor protein (TRAP). It scaffolds Lyn through its PRD domain and then phosphorylates TLR4 to trigger a highly selective pro-inflammatory cytokine response of IL-6 and IL-12p40 production in macrophages. Besides TLR4, SCIMP is also able to directly interact with other TLRs, including TLR2, TLR3 and TLR9. However, the structures of SCIMP and TLR complex has never been explored. Thus, my project aims to characterize the structural basis of SCIMP and TLR4/TLR2 interaction at an atomic level. This will shed light on the underlying mechanism for SCIMP as a universal adaptor in TLR signaling and responses.



13

BIFUNCTIONAL GAR SYNTHETASE/AIR SYNTHETASE AS AN ANTIFUNGAL TARGET

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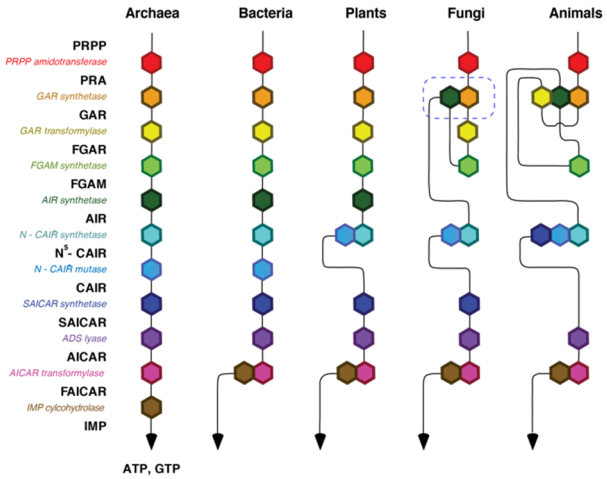
Cryptococcus neoformans is a major fungal pathogen that causes life-threatening systemic mycoses. The current antifungals employed to treat this disease have not changed significantly for the past 25 years despite the high mortality and morbidity. Therefore, there is an urgent need to develop new antifungal drugs. An area of interest for finding new antifungal targets is the purine biosynthesis pathway. Many effective drugs have been developed based on the purine metabolic pathway, however there are limited studies that utilise this pathway as a source of targets for antifungal discovery. The purine biosynthesis pathway consists of eleven sequential enzymatic steps to form IMP, an intermediate for formation of ATP and GTP. Over the course of evolution of the eukaryotes, several gene fusion events have occurred resulting in the formation of bifunctional or trifunctional enzymes in higher order organisms. An example of this is bifunctional GAR synthetase/AIR synthetase, which catalyses steps two and five of the purine biosynthesis pathway. In humans, this has undergone has an additional gene fusion to create a trifunctional enzyme that includes GAR transformylase. The gross differences between the fungal and human enzymes could potentially be exploited in the development of fungal specific inhibitors. The enzyme has been characterised using molecular genetics, enzymology and structural biology techniques. Our work proves that the enzyme is required for *de novo* adenine and guanine production and is essential for virulence in a mouse inhalation model, showing its potential as an antifungal target. Each domain of the protein has been heterologously expressed and crystallised. The GARS domain crystal had a preliminary diffraction of 3 Å and the AIRS domain crystal had a preliminary diffraction of 2 Å, and reveals differences from the human enzyme that could be exploited in antifungal drug development.



14

THE STRUCTURAL DETAILS OF THE INTERACTION OF SINGLE STRANDED DNA BINDING PROTEIN HSS2 (NABP1/OBFC2A) WITH UV-DAMAGED DNA

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Abstract

Single stranded DNA-binding proteins (SSBs) are proteins that are required for all known DNA metabolic event such as DNA replication, recombination and repair. While a wealth of structural and functional data is available on the essential human SSB, hSSB1 (NABP2/OBFC2B), the close homolog hSSB2 (NABP1/OBFC2A) remains relatively uncharacterised. Both SSBs possess a well-structured OB (oligonucleotide/oligosaccharide-binding) domain which is able to recognise single-stranded DNA (ssDNA) and a flexible carboxyl-tail implicated in the interaction with other proteins. Despite the high sequence similarity of the OB domain, several recent studies have revealed distinct differences between hSSB1 and hSSB2.

In this study, we show that hSSB2 is involved in the nucleotide excision repair (NER) pathway and is able to specifically recognise cyclobutane pyrimidine dimers (CPD) that form in cells as a consequence of UV damage. Using a combination of BLI and NMR, we determine the molecular details of hSSB2 binding to CPD-containing ssDNA confirming the role of four key aromatic residues in hSSB2 (W59, Y78, W82 and Y89) that are also conserved in hSSB1. From a molecular perspective, our structural data demonstrate that ssDNA recognition of hSSB2 is highly similar to hSSB1 indicating that one might be able to compensate for the other in initial ssDNA binding events. However, subsequent recruitment of other proteins that play an essential role in the relevant DNA repair pathways is most likely also be determined by the flexible carboxyl-tail and as such will differ between the two SSBs explaining the recently observed functional differences.

15

INTRINSICALLY DISORDERED SEQUENCES OF CAVIN1 DRIVE THE FORMATION OF PLASMA MEMBRANE CAVEOLAE

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Caveolae are spherically curved nanodomains of the mammalian plasma membrane generated by proteins - caveolins, cavins and lipids. Caveolins are integral membrane proteins while cavins are cytosolic peripherally membrane associated proteins crucial to build caveolae in mammalian cells. Cavins possess unique primary and secondary structures, with an alternating distribution of disordered regions (DRs) rich in acidic negatively charged residues and α-helical regions (HRs) rich in positively charged basic residues. The HR domains are involved in binding to the phospholipid membrane while the role of the DRs is unknown. Here we show that the three DR regions of cavin1 are indispensable for caveola formation in cells, with deletions leading to mislocalisation and perturbed interactions with caveolin at the plasma membrane. These defects correlate with an inability of recombinant mutant forms of cavin1 to modify and tubulate artificial membranes *in vitro.* The recombinant cavin1 shows high propensity to self-associate / phase separate in solution at the physiological salt concentration disrupted by the soluble inositol polyphosphate lipid headgroup binding. We thus propose that the fuzzy intermolecular electrostatic interactions between cavin HR – DR domains largely driven by DR sequences and lipid binding interactions through HR domains generate caveolar curvature and form a metastable liquid coat on the cytosolic face of caveolae.

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ELUCIDATION OF NURD AND NUDE PROTEIN STRUCTURE WITH CRYO-EM, AND PURIFICATION OF NURD FROM CHO CELLS

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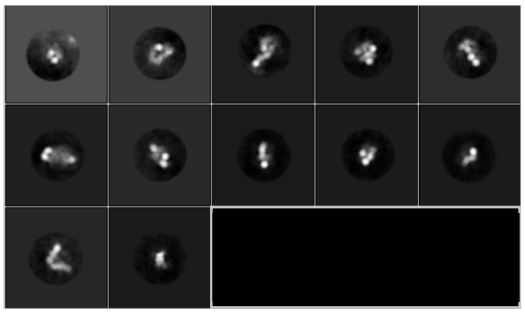
The nucleosome remodelling and deacetylase (NuRD) complex is known to be involved in processes pertaining to DNA repair and cell-cycle progression (Polo *et al*., 2010), cell differentiation (Fujita *et al*., 2003), and chromatin assembly and maturation (Chadwick *et al*., 2009). Structural information of the NuRD complex is limited, researchers instead studying subcomplexes and subunit interactions (Torrado *et al*., 2017). Structural information for many subunits and their paralogs are also incomplete. This study aims to elucidate the structure of the full NuRD complex and the closely related nucleosome deacetylase (NuDe) complex using cryo-electron microscopy (Cryo-EM). Currently, an initial model has been obtained with an estimated resolution of 27.6 Å (2D classes in Figure). It is similar in scale and shape to previously obtained negative staining electron microscopy data (Silva *et al*., unpublished). In parallel, the isolation of the NuRD complex from Chinese hamster ovary (CHO) cells is being pursued. CHO cells are a popular choice of expression system for therapeutic antibodies and other proteins in a secreted state. Isolation of intact NuRD complex from CHO cells previously used for overexpression would provide the cells a dual purpose. Structural and functional analysis of said complex may also provide information on cell-specific and species-specific changes in characteristics such as subunit stoichiometry and isoform distribution.



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PARALOGUE-DERIVED, MUTUALLY EXCLUSIVE NURD COMPLEXES WITH UNIQUE FUNCTIONS

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Chromatin remodelling complexes are tasked with maintaining and manipulating the chromatin accessibility landscape – a process that has inherent and significant consequences should something go awry, for example cancer. Chromatin remodelling complexes are usually composed of a large number of subunits, each with an array of highly related paralogues. It is not fully understood whether the presence of these paralogues is merely an attribute of functional redundancy, or if these paralogues combine in certain ways to create unique chromatin remodelling complexes with distinct functions. The Nucleosome Remodelling and Deacetylase (NuRD) complex is a multi-subunit chromatin remodelling complex that has both ATP-dependent chromatin remodelling activity and histone deacetylase activity. These two activities allow NuRD to alter chromatin density and act as both a transcriptional activator and repressor.

The NuRD complex comprises six canonical subunits, and each of these subunits has a number of highly related paralogues. The implications of combinatorial assembly of NuRD paralogues into distinct complexes are not known, and in order to elucidate the possible diverse functions of distinct NuRD complexes, a better understanding of paralogue composition is required.

Here we use single molecule fluorescence total internal fluorescence (TIRF) microscopy to identify NuRD complex heterogeneity. Our preliminary data show that the core NuRD subunit paralogues MTA1 and MTA2 do not form heterodimers at low nanomolar concentrations, which suggests that they may have a preference to homodimerise to form distinct NuRD complexes. These findings will ultimately help to answer the questions of how NuRD targets specific genes for chromatin remodelling and will elucidate the role of NuRD in diseases such as cancer.

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MACROCYCLIC PEPTIDES AS THE NOVEL CHEMICAL PROBES TO MODULATE RETROMER COMPLEX IN ENDOSOMAL TRAFFICKING

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The evolutionarily conserved retromer complex is a heterotrimer of three subunits, VPS26-VPS29-VPS35, and is a central hub responsible for cargo proteins trafficking in the endolysosomal system. Disruption of retromer-mediated trafficking is also linked to neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD). Given the importance of the retromer complex, little effort has been made to identify small molecules that potentially act as chemical probes or pharmacological chaperones of retromer complex for early stage drug discovery. Here we employed the random nonstandard peptides integrated discovery (RaPID) approach to identify a group of cyclic peptides capable of binding to retromer and its sub-complexes with high affinity. Our biophysical and biochemical analysis suggests that the cyclic peptide Rt-L-04 can significantly enhance the stability of the retromer complex *in vitro* by binding to the VPS26 and VPS35 interface. Further analysis reveals that the presence of Rt-L-04 does not disrupt the retromer complex from binding to cargo adaptors such as SNX3.

In addition to this retromer stabilizing cyclic peptide, we also identified a group of cyclic peptides that bind specifically to the highly conserved pocket of VPS29. Crystal structures show that the cyclic peptides insert into a pocket on VPS29 that is also used by regulatory proteins TBC1D5 and VARP, as well as the bacterial protein RidL. Addition of these high affinity VPS29 targeting cyclic peptides could effectively compete for retromer binding to TBC1D5 as well as RidL *in vitro*. To our knowledge, this is the first molecular probe that could function as retromer inhibitor by blocking VPS29 from binding to accessory proteins and the efficacy of the identified cyclic peptides in cellular models is currently being investigated.

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STRUCTURAL BASIS AND INHIBITION OF HUMAN PARAINFLUENZA VIRUS TYPE 1 HAEMAGGLUTININ-NEURAMINIDASE HOST-CELL ATTACHMENT AND RELEASE

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Human parainfluenza viruses are responsible for a variety of upper and lower respiratory tract illnesses. Human parainfluenza virus type 1 (hPIV-1) in particular, is responsible for causing croup, which commonly requires patient hospitalisation in severe cases. Despite ongoing research efforts, there are no antiviral drugs or vaccines that have been clinically approved to treat hPIV-1 infections to date, which reinforces the urgency for developing novel therapeutic approaches. Current research endeavours are focused on an important surface glycoprotein, haemagglutinin-neuraminidase (HN), which plays a crucial role in virus attachment, fusion and release. In recent years, researchers have attempted to crystallise hPIV-1 HN, in order to conduct insightful, structure-guided investigations to understand the structural basis of host-cell binding and membrane fusion activation. However, all crystallisation attempts to date have not yielded viable crystals. Identifying and characterising the important structural properties of hPIV- 1 HN is a key investigative step in novel antiviral drug discovery. Therefore, the primary objective of this project is to explore new strategies to successfully obtain the three-dimensional structure of hPIV-1 HN. This study will utilise a variety of structural biology techniques such as X-ray crystallography, negative stain and cryo-electron microscopy to resolve the structure of hPIV-1 HN. In summary, this project presents several avenues for gaining novel insights into the structural properties of hPIV-1 HN, in order to assist with the design of future hPIV-1 inhibitors with strong specificity and efficacy.

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MX BEAMLINES CURRENT AND FUTURE DEVELOPMENTS

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The macromolecular crystallography (MX1) and micromolecular crystallography (MX2) beamlines at the Australian Synchrotron provide the structural biology and chemical crystallography communities of Australia & New Zealand with leading edge technology for the acquisition of single crystal data. Recent upgrades to MX1 and MX2 beamlines have seen a step change in both sample and data throughput (through upgrades to both hardware and software). The evolving needs of our user community, in particular, more challenging projects being undertaken has resulted in the production of smaller more weakly diffracting crystals. The demanding nature of these more challenging projects has greatly influenced the direction and design of the new MX3 beamline.

Both MX1 and MX2 now have hybrid photon counting detectors. In 2017, the MX2 beamline installed an EIGER 16M detector, (part funded by the Australian Cancer Research Foundation). This year MX1 became the first beamline in the world to have an EIGER 2 9M detector installed. Datasets on both MX1 and MX2 can now be collected in 36 seconds (or less), which is over 25x faster than was previously possible using the previous ADSC detectors. To make full use of this improvement in collection times it was necessary to undertake upgrades to the sample mounting robot (both software and hardware), the code changes improved sample throughput with sample mount times of 25 seconds. These hardware upgrades in combination with our software development of intuitive graphical user interfaces has greatly improved our capacity to deliver world class equipment for world leading user experiments.

Future development on MX1 includes the installation of a Smargon goniometer which is expected to greatly aid in the development of automated collection strategies for our CX community. Construction of a third MX beamline (MX3) at the Australian Synchrotron has been approved to meet the growing needs of our MX community as part of the BR-GHT project. The drivers behind the conceptual design of MX3 are: making use of small weakly diffracting crystals, improvements in serial crystallography and new/novel methods of sample delivery. The increase in laboratories working in the field of membrane protein crystallography, particularly GPCRs, is driving the need within the community for a smaller focused beam with a higher flux than can be produced by our existing beamlines.

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STRUCTURAL AND BIOCHEMICAL CHARACTERISATION OF THE TIR DOMAINS OF TLRS 7, 8 AND 9 AND THEIR INTERACTIONS WITH ADAPTOR PROTEINS

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Toll-like receptors (TLRs) play central role in triggering innate immune response by recognizing specific pathogen associated molecular patterns (PAMPs) derived from infectious pathogens. TLRs are structurally conserved which consisted of an N-terminal tandem leucine-rich repeats (LRRs) ectodomain, a transmembrane domain and a C-terminal cytoplasmic Toll/interleukin-1 receptor (TIR) domain. TLRs 7, 8 and 9 are a subclass of TLRs localised on the membrane of endosome, which recognise foreign nucleic acid as PAMPs and initiate downstream signalling cascade by the TIR domain via MyD88-dependent pathway.

However, abnormal activation of TLRs 7, 8 and 9 leads to the onset of autoimmune diseases including systemic lupus erythematosus (SLE) and psoriasis. The LRRs ectodomain of TLRs 7, 8 and 9 has been selected as drug design target for autoimmune diseases treatment recently as the availability of their crystal structures, but limited drug effect was stated. In this project, structural studies of the TIR domains of human TLRs 7, 8 and 9 will be performed and the intermolecular interaction between them and adaptor proteins will be investigated. Pure human TLR7-TIR protein has been successfully purified with the final protein concentration of 12.882 mg/ml, the resulting yield was 0.73 mg per liter of ZY media culture. Brown precipitate was observed when crystallisation screening was performed for TLR7-TIR protein, of which the buffer conditions need to be optimised in the next step. Moreover, turbidity assay between TLR7-TIR and MyD88-adaptor-like (MAL) protein has revealed that there might be direct interaction between MAL-TIR and TLR7-TIR, and MAL-TIR might inhibit TLR7-TIR form turbidity, but it has not been convinced. In the future, TLR8-TIR and TLR9-TIR will also be purified and in addition to TLR7-TIR, crystallography studies will be performed. If there is any large signalling assembly complex formed between TIR domains of TLRs 7, 8 and 9 as well as adaptor proteins will be characterised. Hopefully this project will provide a new insight of drug design target for autoimmune diseases.

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INTERACTION AND STRUCTUAL STUDY BETWEEN TNLS AND EDS1 IMMUNE REGULATORY COMPLEX

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Plants have intracellular receptors which recognize plant pathogen effectors, culminating in a severe immune response and localized cell death, known as hypersensitive response (HR). Found at the N-terminus, the Toll-like/interleukin-1 receptor (TIR) domain distinguishes a group of nucleotide-binding leucine rich repeat receptors (NLRs), known as TNLs.

The TIR domain is necessary and sufficient to induce HR. Self-association of the TIR domains (TIR: TIR) has been implicated in plant and animal TIR-containing proteins, as well as recently hydrolytic activity of nicotinamide adenine dinucleotide (NAD^+), an important co-factor found in all living cells. How this relates to the TNL downstream signaling partners enhanced disease susceptibility 1 (EDS1) and respective heterodimer partners senescence associated gene 101 (SAG101) and phytoalexin deficient 4 (PAD4) is currently unknown.

It has been shown previously that TNLs and EDS1/SAG101 interact *in planta* through coimmunoprecipitation experiments during activation of HR. We hypothesize that NAD^+ hydrolysis and oligomerization by the TIR domain facilitates signaling by cooperative assembly formation (SCAF) with the EDS1 immune regulatory complex. We tried pull-down and analytical size exclusion chromatography experiments to observe complex formation between EDS1/SAG101 and the TIR domain of TNL, Resistance to *Pseudomonas Syringae* 4 (RPS4) in the presence of NAD+ and would like to elucidate the structure of the complex through x-ray crystallography.

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PURIFICATION AND REMOVAL OF METALS OF CHLAMYDIAL YTGA, AN IRON BINDING PROTEIN IN *CHLAMYDIA TRACHOMATIS*

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Chlamydia, caused by *Chlamydia trachomatis*, is the most widespread sexually transmitted disease worldwide. As an intracellular pathogenic bacterium, *C. trachomatis* relies heavily on its own nutrient uptake systems to survive in nutrient limited environments. Gram-negative bacteria such as *C. trachomatis* use solute- binding proteins (SBPs) and ATP-binding cassette transporter complexes to transport specific transition ion metals across the periplasm that are required for successful growth and virulence in the host cell. *C. trachomatis* has been shown to utilize YtgA, an SBP, along with its cognate ABC complex YtgBC to sequester iron under iron starvation conditions. Our laboratory has recently resolved the crystal structure of YtgA bound with Fe(III), and binding assays have determined YtgA also binds Mn(II), Fe(II), Fe(III), Co(II), Ni(II), Zn(II) and Cd(II). This indicates a high degree of polyspecificity in comparison to some other metal binding SBPs, such as Zn-binding AdcA and ZnuA from *Strecptococcus pneumoniae* and *Salmonella enterica*, respectively, which are specific for Zn(II). We hypothesize that the binding site of YtgA can accommodate multiple transition metals by utilising different coordination side chains in the metal-binding site according to the binding preferences of the metal. Therefore, we aim to determine the structural differences of YtgA bound to various transition metal ions including Zn(II) and Mn(II) by x-ray crystallography. We demonstrate the process of removing bound metals from the recombinant YtgA protein using a partial unfolding dialysis strategy and confirming the removal of metals via inductively coupled plasma atomic emission spectroscopy which detects the concentration of metals present in the solution. This will generate YtgA protein in its ligand-free form, which will later be complexed with different transition metal ions to acquire homogeneous metal-bound protein for subsequent crystallographic studies.

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STRUCTURAL INSIGHTS INTO BRD3 DRUG DISCOVERY

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Bromodomain-containing protein 3 (Brd3) is a transcriptional regulator that binds chromatin remodelling complexes. The latter function is mediated by Brd3's extraterminal (ET) domain. Disrupting the interaction of the ET domain with chromatin remodellers could lead to the development of novel cancer therapeutics. We have identified promising small molecule binding partners of Brd3-ET using a fragment-based screening approach, and are now in the process of gathering information via NMR to determine the mode of binding. We are also attempting to use x-ray crystallography to speed up the process of gathering structural information on new small molecule inhibitor candidates.

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STRUCTURAL STUDIES ON MOLYBDENUM UPTAKE IN *PSEUDOMONAS AERUGINOSA*

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Pseudomonas aeruginosa are Gram negative bacteria that can survive in adverse conditions. These bacteria are opportunistic human pathogens that cause a large variety of opportunistic infections in immunocompromised patients. These bacteria can thrive in various environmental niches due to their ability to exploit a wide variety of energy sources. In anaerobic conditions, the main form of energy production for *P. aeruginosa* is nitrate reduction. The nitrate reduction pathway consist of four steps by which nitrate is sequentially reduced to dinitrogen. Of specific interest is the rate limiting enzyme complex NarGHI, the first enzyme complex in the pathway that reduces nitrate to nitrite. For the NarGHI complex to function it requires a molybdenum cofactor, Mo-bis molybdopterin guanine dinucleotide. Uptake of molybdenum occurs though the ATP-binding cassette transporter ModABC. ModA is a solute binding protein that is able to bind to periplasmic molybdate and tungstate oxyanions. However, the structure of modA is still unknown. In our experiments, we aim to obtain the crystal structure of ModA in both its unliganded and liganded forms.
Results: We have successfully managed to express and purify ModA using affinity chromatography and size exclusion chromatography. The purity of ModA was confirmed using SDS-PAGE and ESI-MS/MS. We also performed a multiangle laser light scattering coupled with size exclusion chromatography, which shows that ModA exist as a monomer in solution. The protein was crystallised at 10 mg/ml and sent to the Australian synchrotron for X-ray diffraction. We then were able to obtain a high-resolution structure of the ligand-free form of ModA at 2.4 Å and are presently performing model building using molecular replacement and refinement. The current model shows that the structure is similar to other cluster D-III solute binding proteins, consisting of two lobes that are composed of a five-stranded β sheet surrounded by alpha helices connected by two linkers that varies between 2-6 residues long. Currently, optimisation of crystal conditions of its molybdate and tungstate bound form are currently being done.

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STRUCTURAL CHARACTERISATION OF TWO BACTERIAL VIRULENCE FACTORS INVOLVED IN ZINC ACQUISITION IN *PSEUDOMONAS AERUGINOSA*

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There are many different bacterial species that are clinically significant because of their involvement in serious infections and diseases. One such species is the opportunistic zoonotic Gram-negative bacterial pathogen *Pseudomonas aeruginosa*, which has been implicated in patients with cystic fibrosis and with hospital-acquired infections via urinary catheters. Because *P. aeruginosa* is able to survive in many different environmental conditions and is resistant to many types of antibiotics (partially contributed by biofilm formation), this makes it crucial to study the virulence factors that contribute to its pathogenicity and virulence. One such group of virulence factors are the proteins that are involved in zinc homeostasis.

Under zinc limiting conditions, the Zn^{2+}-uptake regulatory (Zur) protein is responsible for regulating zinc acquisition through the up-regulation of several operons. One set of these operons encodes the ATP-binding cassette (ABC) transporter complex, a Zn^{2+}-binding ABC importer responsible for Zn^{2+} uptake from the periplasm. Homology modelling of the solute-binding protein (SBP) ZnuA of the protein complex shows that its predicted structure resembles that of Cluster A-1 of SBPs. Another identified set of operons is the Zur-regulated metallophore (*zrm*) operon, which is responsible for the synthesis, import and export of the zinc opine metallophore, pseudopaline. One of the two enzymes involved in the synthesis of pseudopaline is ZrmB, a nicotianamine synthase-like enzyme with S-adenosyl-L-methionine-dependent aminoalkyl transferase activity.

Given the critical roles these proteins play in zinc acquisition, therefore the main aim of this study is to structurally characterise the two proteins, ZnuA and ZrmB, using X-ray crystallography. The two proteins were successfully expressed and purified as soluble proteins via affinity chromatography and size exclusion chromatography (SEC). SDS-PAGE and mass spectrometry were used to determine the purity of the proteins. The SEC UV chromatograms and multi-angle laser light scattering experiments coupled with SEC suggested that ZnuA existed as a monomer in solution while that of ZrmB is a homodimer. Initial inductively-coupled plasma optical emission spectroscopy (ICP-OES) experiments indicated that the Zn^{2+} metal to protein molar ratio was 1.7:1, suggesting a possible second metal-binding site, though this binding is likely to be transient as the protein was incubated with two-fold excess zinc. More experiments such as the use of native mass spectrometry and isothermal calorimetry (ITC) of the zinc-bound ZnuA would be needed for confirmation. Crystallisation trials of both proteins with sparse matrix screens are currently ongoing. The purified ZrmB protein in the absence of the SAM substrate is prone to aggregation and precipitation at room temperature, suggesting that the SAM substrate may be essential for keeping it stable, with more experiments to be performed to confirm this hypothesis.

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USING DIPTERAN INSECT ASSAYS TO SCREEN ARACHNID VENOMS FOR DISCOVERY OF ORALLY ACTIVE BIOINSECTICIDE LEADS

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The rapid growth of the world population causes an increasing demand for food and therefore calls for proper means of controlling insect pests. Unfortunately, abuse and misuse of chemical insecticides already resulted in environmental concerns and in high resistance rates amongst many insect pests. Alternatively, some spider-venom toxins are considered as environmentally-friendly bioinsecticides given their advantages of being fully biodegradable, highly potent and phyletically selective. However, the fact that spider toxins evolved to be active upon injection into their prey has questioned a potential use as bio-insecticides due to their presumed lack of oral activity. Therefore, the aims of this study were to develop an assay for efficient and easy screening of oral insecticidal activity of crude spider venoms, and to isolate and characterise new orally active toxins. Fruit flies (*Drosophila melanogaster*) and sheep blowflies (*Lucilia cuprina*) were selected for screening 56 arachnid venoms. 71.4% of these venoms caused 50% or higher mortality in fruit flies, whereas 30.4% were lethal to blowflies at oral doses of 1 or 30 µg/fly, respectively. We found that a high oral activity was caused by the two orally active theraphosid toxins Pi1a and Avsp1a. In fruit flies the 43-residue peptide Pi1a shows an oral LD_{50} of 47.2 nmol/g, whereas the 39 residue Avsp1a exhibited an oral LD_{50} of 28.5 nmol/g. We also used these assays to compare the oral and injection activity of four well-known insecticidal spider toxins (Hv1a, Hv1c, Dc1a and Ta1a). In regards to their oral toxicity, Dc1a was most potent in both dipteran species with a LD_{50} of around 20 pmol/g, whereas Hv1a and Hv1c were only active in blowflies and Ta1a completely lacked oral activity. Our results indicate that oral insecticidal activity is more widespread in arachnid venoms than previously assumed and we can also conclude that some orally insecticidal spider toxins exhibit phyletic specificity. Further studies will focus on the mechanism of action and structure-function relationships of Pi1a and Avsp1a, aiming at rational engineering of peptides with improved potency and selectivity towards pest species as potential leads for bioinsecticides.

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IN SILICO MODELS FOR EXTRACELLULAR GLYCOPHORIN A (GYPA) AND B (GYPB) STRUCTURE

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Background
Glycophorin A (GYPA) and B (GYPB) are glycoproteins found on the surface of red blood cells (RBCs) making up the MNS blood group system. There are 49 currently known clinically significant antigens associated with MNS. Understanding the structural basis for antigen-antibody recognition of glycophorins may lead to improved blood typing reagents.

There are no existing X-Ray crystallography models of the extracellular domains of GYPA and GYPB due to their high level of glycosylation (Chang et al., 2007; Lee, Fusco, & Saphire, 2009). Ab initio structural modelling method was chosen to predict the structures of GYPA and GYPB.

Method
All Molecular Dynamics (MD) simulations were preformed using the GPU version of GROMACS 2019.1 on the Wiener HPC cluster at the University of Queensland, with the GROMOS 54A7 force field for modelling protein structures. Each protein was placed in a cubic periodic box and solvated with SPC water model. Stability was analysed using Root Mean Square Deviation (RMSD) on backbone atoms, and clustering of the relevant combined MD simulation trajectories in which each 200 ns trajectory contained 10,000 frames.

Results
The preliminary results indicate that the extracellular domains of both GYPA and GYPB are intrinsically disordered proteins based on 6 models for GYPA and 5 models for GYPB. It is observed that the structure of the Exon 3-4 junction in GYPA converges to a common structure across models obtained by different methods.

Conclusion and Future Work
The models from this study will serve as the foundation for future work regarding the overall structure of GYPA and GYPB (including the extracellular, trans-membrane and intracellular domains) and will be further refined as more experimental data is produced.

Further work will be performed on the transmembrane region of GYPB such that the GYPA and GYPB extracellular regions can be combined to form a hybrid GYPA/GYPB monomer. GYPA and GYPB homo- and hetero-dimers will also be investigated.

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GLUTARALDEHYDE CROSSLINKING CAN BE COMBINED WITH SEC-MALLS TO ESTIMATE THE MOLECULAR MASS OF UNSTABLE PROTEIN COMPLEXES

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Glutaraldehyde is a homo-bifunctional cross-linker that can react with a wide variety of protein functional groups including amines, hydroxyls, thiols and imidazole. Despite being one of the most commonly used cross-linkers in protein biochemistry, its exact behaviour in aqueous solution is complex and it forms very heterogeneous crosslink populations with widely ranging molecular masses.

Proteins often work in multi-subunit molecular machines. The molecular weights and stoichiometry of protein complexes are usually one of the major goals of many protein biochemistry projects. However, the isolation and manipulation of these complexes is often fraught with stability issues, and this complicates their analysis.

We propose the use of the established GraFix protocol, which uses glutaraldehyde, in combination with SEC-MALLS to determine the molecular mass of protein complexes. To address the heterogeneous nature of glutaraldehyde crosslinks, we used standard control proteins to estimate that our GraFix protocol adds ~540 Da to the mass of a protein per lysine residue.

To test our hypothesis, we have applied it to the Nucleosome Remodelling and Deacetylase (NuRD) complex and one of its subcomplexes — the NuDe complex. SEC-MALLS of both the NuRD and NuDe complex yielded masses that were within 10% of their predicted masses (based on extensive quantitative mass spectrometry data).

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STRUCTURAL INSIGHTS INTO THE CONFORMATIONAL CHANGE OF COMMD PROTEINS USING X-RAY CRYSTALLOGRAPHY.

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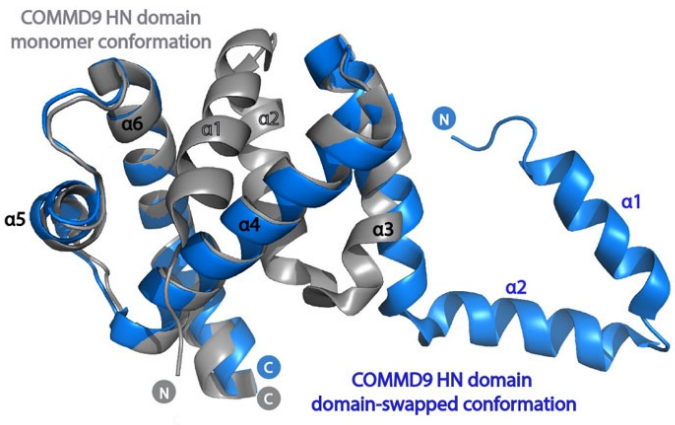
Our lab is interested in understanding how membrane-embedded proteins are sorted between different cellular organelles. We use various methods to study the structures of proteins involved in membrane trafficking, including X-ray crystallography to determine how the protein's atoms are arranged in three dimensions. I will highlight some of the work that I am doing as part of my MPhil to study the structures of a family of proteins called COMMDs (Copper Metabolism Murr1 Domain-containing).



The COMMD proteins are a highly conserved family of proteins with central roles in intracellular membrane trafficking and transcription. They form oligomeric complexes with each other and act as components of a larger assembly called the CCC Complex, which has been shown to regulate the trafficking of key receptors including ion transporter ATP7A, the lipoprotein receptor LDLR, and the signaling receptor NOTCH2. However, the stoichiometric composition and underlying assembly of COMMD proteins within the Commander complex remains unclear.

Using X-ray crystallographic techniques, we have determined the structures of several α-helical N-terminal (HN) domains of COMMD proteins and my studies suggest that the HN domains of specific COMMD proteins can adopt alternative structures that might be important for the way that they form large complexes in the cell required for membrane trafficking. As shown in Figure; Commd9 undergos significant conformational remodelling via domain swapping of alpha helices 1 and 2. This domain swap is likely regulated by protein phosphorylation of a serine rich "hinge" region between a-helix 2 and a-helix 3, with mutations in this region preventing high order assembly, a characteristic of the domain swapped protein. These studies suggest that the HN domain of specific COMMD proteins may assist in the assembly of the higher ordered CCC complex. Overall this study provides novel insight into the mechanism of COMMD HN domain swap characteristics.

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STRUCTURAL AND FUNCTIONAL CHARACTERISATION OF A PYRIMIDINE SYNTHESIS COMPLEX FROM YEAST

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De-novo pyrimidine biosynthesis is conserved in all domains of life. This process is carried out by a series of enzymatic reactions that build up the pyrimidine ring from the small molecule precursors ammonia, bicarbonate, ATP and aspartate (Denis-Duphil, 1989). In both bacteria and eukaryotes this process involves the production of the metabolic intermediate carbamoyl phosphate by liberating ammonia from glutamine with a glutaminase (GLN), followed by a ATP hydrolysis-coupled conjugation of ammonia and bicarbonate to form carbamate in the first active site of a darbamoyl phosphate synthetase (CPS) (Charlier *et al.*, 2018). Carbamate is then phosphorylated by ATP in the second site of CPS, followed by conjugation of the resulting carbamoyl phosphate with aspartate by aspartate transcarbamolyase (ATC) to form carbamoyl aspartate (Jones, 1980). This intermediate is then subject to a ring closing reaction to form dihydroorotate by dihydroorotase (DHO), creating the ring that is then subsequently modified by dehydrogenation, addition of phosphoribosyl pyrophosphate and decarboxylation to form uridine monophosphate. In bacteria such as E.coli the enzymes for each of the four first steps in this pathway are encoded separately (Davidson *et al.*, 1993), however in animals and fungi the GLN, CPS, ATC and DHO enzymes are fused into a single large polypeptide massing approximately 240 kDa in S. cerevisiae, in which it is known as URA2 (Denis-Duphil, 1989:2). In mammals this gene is called CAD (Lee *et al.*, 1985). Animals harbour all four activities in CAD, however in fungi the DHO module of the multienzyme is non-functional; its enzymatic role has been replaced by a separately encoded DHO called URA4 (Denis-Duphil, 1989:4). In this work we describe efforts towards characterisation of the structure and function of the GLN module of S.cerevisiae URA2 and further work on expressing the full-length URA2 multienzyme in Pichia pastoris for characterisation by electron microscopy.

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ASSEMBLY OF THE NUCLEOSOME REMODELLING AND DEACETYLASE (NURD) COMPLEX IN GATAD2B ASSOCIATED NEUROLOGICAL DISORDER (GAND)

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The hallmarks of GATAD2B Associated Neurological Disorder (GAND) are severe intellectual disability, impaired speech development and distinct facial features such as broad nasal tip and short philtrum. The disease is connected to *de novo* mutations in the highly conserved CR1 and CR2 regions of the protein GATAD2B. GATAD2B is a component of the nucleosome remodelling and deacetylase (NuRD) complex where it acts as 'bridge' linking the MBD and CHD subunits.

The NuRD complex functions as a regulator of chromatin structure and it is essential for embryonic development, chromatin assembly and DNA damage repair. Each component of the NuRD complex exists as a number of paralogues in mammals, and this feature is thought to contribute to its diverse function. Using pairwise co-immunoprecipitation experiments, we show that five *de novo* mutations in GATAD2B affect binding to NuRD components CHD3/4/5 and MBD2/3, suggesting a possible disease mechanism in which GAND affects NuRD assembly.

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STRUCTURAL BASIS OF TIR DOMAIN ASSEMBLY FORMATION IN THE TOLL-LIKE RECEPTOR TRIF-DEPENDENT PATHWAY

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Toll-like receptors (TLRs) are pathogen/damage recognition receptors that detect pathogens directly or detect the damage caused by them. Upon detection the TLRs recruit down-stream adaptor proteins important for a mounting an immune response. TLRs can signal through either the MyD88-dependent signalling pathway or the TRIF-dependent (MyD88-independent) signalling pathway. The MyD88-dependent pathway is the primary driving force for the early NF-κB response, a broad-spectrum response for early immune signalling. While TRIF-dependent signalling is involved in the late NF-κB response, a pathway that is linked to cell-death signalling pathways and type-1 interferon expression.

Previous work in our lab has shown that the adaptor protein MAL (PDB ID 5UZB) of the MyD88-dependent pathway undergoes assembly formation into a filament *in vitro*. The filament itself is cylindrical with a diameter of ~ 300 Å with a C6 symmetry composed of 12, two stranded proto-filaments. Mutations in the inter-strand interface between subunits generally disrupts the function of MAL *in vivo*.

TRAM Filament formation was observed using Electron microscopy, showing a highly flexible actin like filament. This filament is an ellipsoid with a diameter of ~ 90 Å on wider axis and ~ 45 Å on narrow axis. Averaging of the segments reveal a featureless cylinder with strong layer lines, indicative of a helical protein. 2D-classification of the helical segments shows a single-start filament like a crystallographic two-one screw axis (2-sub$_1$) symmetry. The displacement between individual protein subunits appears to be consistent with similar layer lines being generated from various classes. The in-plane-rotation (rotation along the filaments axis) appears to vary between individual classes by a factor of several degrees, possibly caused by torsional disorder in the ice.

Some classes exist with a very slow twist which when averaged via 3D-classification generates a low-resolution map which can fit the TRAM^{TIR} NMR structure but with dubious fitting. The poor fitting is primarily due to variable twist along the helical axis (2nd type para-crystalline disorder) which prevents accurate averaging of the high-resolution information. To improve this structure, I am attempting to exclude various views where the in-plane-rotation varies too much from the standard. One such class exists in my data-set where the twist is almost 180 ° leading to a very flat class, this class could in theory be averaged to generate a high-resolution class with the interfaces intact.

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IL-1R8 (SIGIRR) REGULATES TLR4 SIGNALING

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Interleukin-1 receptors (IL-1Rs) are membrane receptors that are vital to innate host defense against infection, allergic and non-allergic inflammation, injury and stress. IL-1R8, also known as single immunoglobulin interleukin-1 receptor-related protein (SIGIRR) is an inhibitory receptor which regulates signaling of IL-1Rs and other TIR-domain containing receptors called Toll-like receptors (TLRs). It is characterized by a single extracellular immunoglobulin-like domain, an intracellular TIR domain and a 95 residue long cytoplasmic tail. The TIR domain is a protein:protein interaction domain which plays a critical role in cytoplasmic signaling by forming TIR:TIR interactions between receptors and cytoplasmic adaptor proteins to mediate downstream signaling leading to pro-inflammatory/anti-inflammatory responses. Evidence from gene targeting experiments suggests that the intracellular TIR domain of IL-1R8 alone is sufficient to inhibit LPS-induced TLR4 signaling. Our current research focuses on the structural and functional characterization of the TIR domain of IL-1R8 and the mechanism of how IL-1R8 regulates TLR4 signaling.

The TIR domain of human IL-1R8 (164-311) was cloned, expressed and purified using *E.coli* host system. X-ray crystallization and protein-NMR studies are in progress to determine the structure of the TIR domain. It is well established that the TIR domain promotes TLR signaling through TIR:TIR interactions between receptor: receptor and receptor: adaptor proteins. Because TIR domain alone was shown to inhibit TLR4 signaling, we predict that the inhibition is due to interactions between either IL-$1R8^{TIR}$:$TLR4^{TIR}$, IL-$1R8^{TIR}$:$MyD88^{TIR}$, or IL-$1R8^{TIR}$:MAL^{TIR}, which are the receptor and adaptor proteins involved in TLR4 signaling. Turbidity assays, negative-stain electron microscopy (EM) and single-molecule fluorescence spectroscopy (SMFS) analysis indicate a potential interaction between IL-$1R8^{TIR}$ and MAL^{TIR}. MAL^{TIR} self-assembles and forms characteristic long filaments at 60 μM (cryo-EM structure solved), but in presence of IL-$1R8^{TIR}$, MAL^{TIR} forms morphologically different filaments at a much lower concentration (30 μM), suggesting IL-$1R8^{TIR}$ and MAL^{TIR} form co-filaments. We are currently focusing on solving the 3D structure of MAL^{TIR}/IL-$1R8^{TIR}$ filaments using negative-stain EM and cryo-EM to obtain molecular insights into the interaction interfaces and binding sites of IL-$1R8^{TIR}$ and MAL^{TIR}. Eventually, this can lead us to a detailed understanding of how IL-$1R8^{TIR}$ inhibits TLR4 signaling by targeting MAL^{TIR}.

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THE NUCLEOSOME REMODELLING AND DEACETYLASE (NuRD) COMPLEX HAS AN ASYMMETRIC, DYNAMIC AND MODULAR ARCHITECTURE

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The NuRD complex is essential for normal development and regulates both gene transcription and DNA damage repair. We have used structural, biophysical and biochemical data to define the architecture of the native mammalian complex. We showed that the complex displays considerable dynamics and we identified stable subcomplexes within NuRD, showing that the full complex is composed of two parts with separable enzymatic activities. A pseudo-symmetric deacetylase module comprising MTA, HDAC and RBBP subunits; whereas MBD, GATAD2 and CHD subunits form an asymmetric 1:1:1 arrangement with remodelling activity. The previously enigmatic GATAD2 both controls the asymmetry in the complex and recruits the ATP-dependent CHD remodeller. Taken together, our data define the architecture of the intact NuRD complex, revealing its structural dynamics and functional plasticity.

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CRYO-EM ANALYSIS OF FILAMENTOUS CHAPERONINS

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Thermophilic Factor 55 (TF55, rosettasome) is a type II chaperonin occurring in *Sulfolobus*. Filaments composed of TF55 have previously been observed in vivo and in vitro, but the nature of the interactions between successive subunits has not been established. We have used cryo-EM to image a TF55 β subunit preparation isolated from *Sulfolobus solfataricus*, revealing filaments reminiscent of those seen in *Sulfolobus shibatae*. Averaging methods reveal that the oligomerisation surface relies on overlap of the helical protrusions, which matches contacts seen in previous crystallographic studies. Our findings further confirm hypotheses that filaments play a role in the regulation of chaperonin activity or Archeal cytoskeleton.

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STRUCTURAL INSIGHTS INTO THE BACTERIAL FLAGELLUM

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The bacterial flagellum is a complex nanomachine that allows bacteria to swim in liquid environments and swarm on solid surfaces. This nanomachine is comprised of over 20 different proteins, which assemble to form a long filament, rod, hook, export apparatus and rotary motor. Despite decades of study, the three-dimensional structures of some of these proteins have remained poorly resolved. Using cryo-electron microscopy we have obtained high-resolution structural information about protein complexes within the flagellar motor. This information gives further insight into the function and assembly of the bacterial flagellum.