QMB Proteins Abstracts

P1: Tracking how mutant Huntingtin assemblies into inclusions reveals a Pyrrhic victory for survival

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Two popular models for how mutant Huntingtin exon 1 (Httex1) aggregation into inclusions relates to pathogenesis involve seemingly contradictory mechanisms. In one model, inclusions are adaptive by sequestering the proteotoxicity of soluble Httex1. In the other, inclusions compromise cellular activity from proteome co-aggregation. Via a biosensor of Httex1 conformation in mammalian cell models, we discovered a mechanism that explains this contradiction and I will describe the work here. In essence, we found newly-formed inclusions are comprised of disordered Httex1 and ribonucleoproteins. As inclusions matured, Httex1 reconfigured into amyloid, and other glutamine-rich and prion-domain containing proteins were recruited. Soluble Httex1 caused a hyperpolarized mitochondrial membrane potential, increased reactive oxygen species and promoted apoptosis. Inclusion formation triggered a collapsed mitochondrial potential, cellular quiescence, and deactivated apoptosis. We propose a revised model where inclusion sequestration of soluble Httex1 is pyrrhic: inclusions remove the trigger for apoptosis but also co-aggregate other proteins that stalls cellular functioning. This leads to metabolic quiescence and slow death by necrosis.

P2: Viruses inhibit host cell necroptosis by forming decoy amyloid fibrils

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Stable protein fibrils that have an amyloid substructure are utilized by fungi, viruses and bacteria to facilitate infection and for protection against host cell defenses. The large DNA viruses, cytomegalovirus and herpes simplex virus, evade host defense pathways by expressing inhibitors of the host programmed cell death pathways apoptosis and necroptosis. In murine cytomegalovirus, the protein M45 inhibits host necroptosis. In herpes simplex virus, the protein ICP6 plays a similar role. We show that the RIP homotypic interaction motif (RHIM) within these proteins renders them amyloidogenic and that M45 and ICP6 RHIM-containing domains form homomeric amyloid fibrils with hallmark physical characteristics. Additionally, we have used a wide range of biophysical techniques, including single molecule fluorescence studies, to demonstrate that these proteins are able to form hetero-oligomeric amyloid fibrils through co-assembly with the receptor interacting kinases RIPK1 and RIPK3 and the cytoplasmic viral sensor ZBP1, which all contain RHIMs. RIPK1 and RIPK3 have previously been shown to form a functional hetero-amyloid complex that signals for cell death by necroptosis. The M45 protein displays different affinities for RIPK1 and RIPK3. The incorporation of the viral protein into the host protein amyloid fibrils alters the structure and stability of the amyloid complexes. Our results demonstrate that the ability of the viral protein M45 to form competing “decoy” amyloid fibrils with host RHIM-containing proteins underlies suppression of RIPK-induced programmed necroptosis by this virus.

P3: Recognition by host nuclear transport proteins drives viral protein disorder-to-order transition

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Hendra virus (HeV) is a highly pathogenic zoonotic paramyxovirus that causes severe disease and a high incidence of fatality in infected humans. Despite recurrent outbreaks and potential for human lethality, no vaccine or antiviral agent is available to prevent or treat human HeV infection. Key to HeV pathogenicity is the co-transcriptional mRNA editing of the phosphoprotein (P) gene to generate additional mRNAs encoding the V and W proteins. The V protein modulates the host response to infection by targeting numerous host proteins. New host proteins are frequently being discovered, including those involved in nuclear trafficking. By combining in vitro and in vivo analyses, we show both nuclear import and export receptors are amongst those targeted by HeV V, and play a role in infection. Circular dichroism (CD) spectroscopy and small angle X-ray scattering (SAXS) were used to investigate changes in the folded state of V upon binding to its nuclear transport partners. Fluorescent cell microscopy and immunoprecipitation analysis was used to determine the cellular localisation and binding partners of both wild-type and mutant versions of HeV V. Structural analysis reveals HeV V is inherently disordered and gains structure upon binding its target nuclear transport receptors; a trait that offers numerous functional advantages. Studies with import and export inhibitors suggest a previously unknown role for HeV in the nucleus during infection.

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P4: Understanding the mechanism of ester bond formation in bacterial adhesins

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Most globular proteins are thought to be only marginally stable. The main stabilising forces for a protein scaffold are considered to be the numerous weak interactions forming between the side chains and the back bone of a folded polypeptide chain. Despite the diversity of amino acid side chains, only cysteines were known to form covalent (disulfide) bonds that stabilise protein scaffolds.

The discovery of isopeptide bonds in the surface protein of the Gram-positive bacterium Streptococcus pyogenes changed this simplistic view (Kang et al. 2007). Isopeptide bonds are formed auto-catalytically between lysine and asparagine or aspartate residues that are brought together in a hydrophobic environment during protein folding. More recently, new intramolecular crosslinks formed between threonine and glutamine side chains (ester bonds) were discovered in the surface protein Cpe0147 of another Gram-positive bacterium, Clostridium perfringens (Kwon et al. 2014).

Similarly to the isopeptide bonds, ester crosslink formation appears to be an auto-catalytic reaction, in this case with a mechanism analogous to that of a serine protease utilising a catalytic triad of threonine, histidine, and aspartate residues. However, unlike the serine protease mechanism where a water molecule attacks and hydrolyses an acyl intermediate to regenerate the active site (and produce a cleaved peptide), the ester bond in the Cpe0147 is stable and does not react further.

We have begun to elucidate the full mechanism of ester bond formation in the Cpe0147 protein and to define the structural and chemical factors involved in this autocatalytic reaction. To do this, we are following ester bond formation via Nuclear Magnetic Resonance (NMR), and are probing the steric and chemical determinants of bond formation via site directed mutagenesis.

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Kwon, H., Squire, C. J., Young, P. G., & Baker, E. N. (2014). Autocatalytically generated Thr-Gln ester bond cross-links stabilize the repetitive Ig-domain shaft of a bacterial cell surface adhesin. Proceedings of the National Academy of Sciences of the United States of America, 111(4), 1367–72. http://doi.org/10.1073/pnas.1316855111

P5: New Strategies for Immunotherapy

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We describe a novel platform for the clonal specific modulation of disease-relevant T cells. Our approach manipulates antigen-specific (i.e., clonal) lymphocyte populations by covalently linking single chain peptide-MHC (sc-pMHC) and costimulatory molecules in a manner that recapitulates the proximity, orientation and overall organization experienced at the immunological synapse. The sc-pMHC unit serves to selectively target distinct T cell clones for the delivery of a modulatory domain that can represent any potential costimulatory function. These constructs are generated as Fc-fusion proteins (i.e., IgG) for enhanced avidity and stability. This combined targeting:modulation construct is referred to as **synTac** (artificial immunological **Syn**apse for **T**-cell **Ac**tivation). The extreme specificity associated with these reagents promises to eliminate the extensive side effects associated with immunotherapeutics currently in use, and the highly modular design supports a wide range of indications and therapeutic mechanisms via substitution of the disease relevant peptide epitope and comodulatory components, respectively.

P6: The evolution of MACPF/CDC toxins: multiple assembly pathways for multiple targets

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Members of the Membrane Attack Complex / Perforin-like / Cholesterol Dependent Cytolysin pore forming protein superfamily (MACPF/CDC) perform key but diverse roles throughout all kingdoms of life. Some of these include bacterial pathogen factors, fungal defense toxins, animal venoms and animal immunity effectors. Two key MACPF/CDC toxins used by vertebrates to target bacteria include the terminal complement pathway complex, the Membrane Attack Complex (MAC), and MPEG, a protein found within the phagosomes of macrophage. Numerous high-resolution X-ray crystal structures and low â€“resolution single-particle cryo-EM (SP cryo-EM) pore structures for MACPF/CDC proteins have been determined including the bacterial CDC toxins, fungal pleurotolysin toxin and immune factor perforin. These structures support a mode of pore formation that proceeds via a membrane-dependent oligomerisation of a pre-pore. The pre-pore then undergoes a extensive conformational changes that leads to the insertion of a giant amphipathic Î²-barrel into the lipid membrane. Depending upon the family member, the pore is made of 13-50 subunits leading to a giant pore of between 80-300 Angstrom. This can permit the passive transport of protein in a folded state across the cell membrane. However, new structural research challenge the dogma of MACPF/CDC assembly pathway particularly where the pore forming protein needs to target a variety of surfaces. Firstly, the poly-C9 structure shows how the C9 component of the MAC can assemble into a pore using a membrane-independent pathway. Secondly the high resolution structure of the macrophage phagosome toxin, MPEG, provides insight into how a soluble prepore may overcome the variety of surfaces needed to be targeted. Overall these structures show in both the MPEG and polyC9 pore assembly pathways is mediated via the ancillary domains not the common MACPF/CDC pathway.

P7: Scratched, then sniffed?
Exploring the role of chemoreceptors in host invasion.

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Anyone who has swatted a hungry mosquito away from their arm, or followed their own nose towards fresh coffee, will appreciate the usefulness of chemosensory cues for insects and mammals. Microorganisms also display surprisingly sophisticated sensory behaviours. Motile bacteria are attracted by certain chemicals and repelled by others, a behaviour that enables them to navigate towards favourable niches for growth and survival. This process – chemotaxis – is used by many plant pathogens, to navigate over the plant surface in order to locate potential entry sites. However for the vast majority of bacteria, neither what they detect, nor how they detect it, is understood.

We are using a range of microbiological and biophysical techniques to study the chemosensory repertoire of plant pathogens such as *Pseudomonas syringae* pv. *actinidiae* (*Psa*). *Psa* is motile, and invades kiwifruit plant tissues through natural openings (*e.g.* stomata, lenticels), or via lesions or wounds. It also has an unusually complex chemosensory system, with 43 predicted chemoreceptors. Our studies of these receptors are providing insights into their structural and functional diversity, as well as their role in host invasion.

P8: Architecture of a large DNA virus as revealed by cryo-electron microscopy

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Megavirales are a group of extremely complex double-stranded DNA viruses with intriguing three dimensional architectures. Numerous structural proteins and the large dimension of the capsid makes them a difficult task for structural biologists. Their basic capsid structure shows a combination of pentasymetrons (PS) and trisymetrons (TS) which assemble into an icosahedral capsid protecting a membrane enclosed genome. We used cryo-electron microscopy and phase plate cryo-electron tomography to study the structure of Wiseana Iridovirus (WIV), one of the smallest members of the large DNA virus family. WIV has a 212 kbp genome and infects insects whose larvae become iridescent upon viral capsid accumulation. Our analysis shows that WIV has a T=147 capsid assembled from PS (formed by 31 capsomers) and TS (formed by 55 capsomers). A long flexible fibre emanates from the middle of each trimeric capsomer. The major capsid protein has the expected double jelly-roll structure with prominent loops at the exterior. Tomograms show that the membrane interacts with several TS via specific proteins occupying only part of the interior of the capsid. We also obtained a structure of an empty capsid at subnanometer resolution. Comparison between the two structures suggests a mechanism for capsid assembly. Our results suggest that the TS are capable of independent assembly in the absence of membranes, while the PS are added to preformed capsids together with a chain of zipper proteins which stabilize the capsid.

P9: Lattice Light Imaging of intracellular events - examples from endocytosis and trafficking.

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So far, attempts to follow in real time endocytic events in cultured cells have been limited to events occurring on the ventral (attached) surface or relatively small regions of the dorsal (free) surface. The advent of the powerful lattice light-sheet microscope (LLSM) based on ultrathin light sheets for real time 3D imaging with single molecule sensitivity (Chen *et al.*, 2014) provided us with the unique opportunity to simultaneously explore the dynamics of cargo uptake mediated by the clathrin-dependent and clathrin-independent routes within the same cell. Here, we used LLSM for direct visualization and molecular counting during endocytosis of fluorescently tagged transferrin (canonical cargo for the clathrin endocytic route) and Shiga toxin (a cargo for the clathrin-independent route) in genome-edited cells expressing fluorescently tagged chimeras of clathrin light-chain A, the endocytic adaptor AP-2, and endophilin (constituent of the clathrin-dependent and independent routes). Further, the work under progress on intracellular trafficking will be briefly presented.

P10: A fly-casting, thread, bend and barb mechanism ensures accurate cleavage of branched DNA molecules

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Flap endonucleases (also known as 5' nucleases) are essential for Viability in all organisms from bacteria to man. They are also widely used in clinical diagnostics e.g. In Taqman, genotyping and Realtime PCR. These enzymes cleave branched DNA structures to produce a ligatble nick that can be restored to the canonical double helix. Such branched DNA structures occur during replication and are known as Okazaki fragments. Here, an RNA primer is extended by DNA polymerase but must eventually be strand displaced by an upstream fragment resulting in a 5' single stranded flap. It has long been postulated that the single stranded end might thread through a hole in the flap endonuclease. This has proved continua and direct evidence has been lacking until recently. We used "metal mimic mutagenesis" to enable crystallisation of a metal-enzyme-DNA complex. Structure determination and biochemical studies show that DNA threads through the protein provinding mechanistic insight into an essential biological process.

P11: Mechanistic studies of how the pseudokinase, MLKL, is activated and kills cells by necroptosis

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In 2012, Mixed lineage kinase domain-like (MLKL), a catalytically-dead cousin of conventional protein kinases, termed a pseudokinase, was implicated as the key effector in the programmed necrosis (or necroptosis) cell death pathway. This pathway has been implicated in innate immunity, the pathogenesis of inflammatory diseases, and tissue injury arising from ischemic stroke or acute kidney injury. As a result, an improved fundamental knowledge of the pathway is of enormous interest as we and others look to inhibit the pathway therapeutically.

Our work has focused on the molecular mechanism underlying MLKL activation and how it kills cells. Unexpectedly, these studies have unveiled MLKL's pseudokinase domain as a mediator of many functions: as a molecular switch, as a protein interaction domain for recruiting co-effectors, and as a suppressor of the killer function of the adjacent four-helix bundle domain. Here, we present our dissection of the molecular mechanism underlying MLKL activation and cell permeabilisation using structural, biochemical, biophysical and cellular biology approaches.

P12: Tales of sugary delights

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Sialic acids comprise a varied group of nine-carbon amino sugars that are widely distributed among mammals and higher metazoans. Commensal and pathogenic bacteria that colonise heavily sialylated niches (e.g. the mammalian respiratory tract and gut) can scavenge sialic acid from their surrounding environment and metabolised as a carbon, nitrogen and energy source—that is, they eat your glycogonjuates for breakfast. Sequestration and degradation of sialic acid involves specific amino sugar transporters responsible for the import into the bacterial cell and five catabolic enzymes that successively degrade sialic acid. Regulation of this pathway is achieved at the transcription level by specific repressor proteins.

I will describe two stories: the first delineates the mechanism by which the enzyme *N*-acetylmannosamine-6-phosphate 2-epimerase catalyses epimerisation using a low-barrier hydrogen bond; the second uncovers the mechanism of sialic acid gene regulation.

P13:

Charlie Bond

P14: Insights into the multiple activities of a primordial-like enzyme from *Thermotoga maritima*

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In contrast to our conception of today's numerous, specialist enzymes, primordial enzymes are proposed to have been few in number and with relatively broad functionality. In order to discover primordial-like enzymes in extant species we conducted bioinformatic searches of over 1000 bacterial genomes in the NCBI database.[1]

Our search focused on identifying candidates that may act as a bifunctional cystathionine β-lyase and an alanine racemase,[2] involved in the pathways of methionine and cell wall biosynthesis respectively. We characterised the activities of the CBL enzymes from three species, in two of which we discovered a third activity – glutamate racemisation.[1] This was significant as all known glutamate racemases are co-factor independent, whereas the multifunctional enzymes use pyridoxal 5'-phosphate (PLP).

The CBL from one of the species we investigated, *Themotoga maritima*, was also able to promiscuously catalyse the racemisation of several other amino acids. The interesting evolutionary trajectory and range of substarates accepted by this enzyme led us to look at it in greater detail. We used error-prone PCR to generate a library of variants and screened for improved alanine racemase activity. We identified a triple mutant that exhibited a 44-fold increase in alanine racemase activity but had unchanged glutamate racemase activity and greatly decreased CBL activity, relative to the wild type. We used X-ray crystallography to solve the structures of the triple mutant to 2.6 Å and the wild type CBL to 2.0 Å, and also to 2.6 Å in the presence of an L-alanine phosphonate analogue. The comparison of these structures will provide us with an insight into the activity trade-offs that we have observed and into the evolution of specialist enzymes from multi-functional primordial-like ancestors.

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P15: Structure of the Tersinia entomophaga ABC toxin complex at near-atomic resolution

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ABC toxins are large (1.5-2.5 MDa), tripartite pore-forming toxins secreted by a wide range of Gram positive and Gram negative bacteria. They are the predominant virulence factors in many insecticidal bacteria, where they characteristically trigger apoptotic cell death by delivering cytotoxins that target the actin polymerization pathway to selectively targeted host cells. Genes encoding related toxins are also found in bacterial pathogens of significance to humans although their role in virulence remains unclear. Recent advances in single particle cryo-electron microscopy have allowed the mechanism by which prototypical bacterial ABC toxin systems package, translocate and deliver potent cytotoxins to targeted inter-cellular destinations to be visualised in near-atomic detail. Here we will focus on insights obtained from the first near-atomic resolution structure of a representative type II toxin – the 2.3 MDa YenTc from the insect pathogen *Yersinia entomophaga*. Our results suggest YenTc has a distinctive mechanism of cell surface recognition and provide the first clues as to the identity of candidate ligands that direct their interaction with epithelial cell surfaces. We show that YenTc is capable of forming a transmembrane pore, but in contrast with previously characterized type I toxins from *Photorhabdus luminescens*, formation of the pore is only weakly stimulated by extreme pH. Our results therefore challenge the prevailing paradigm of how pore-formation is triggered physiologically.

P16: Uncovering mechanisms for the self-assembly of supramolecular protein structures with artificial DNA templates.

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Large multi-subunit protein complexes self-assemble spontaneously yet do not prematurely form unwanted aggregates. Static snapshots of intact complexes or component parts provide little insight into how this occurs. We combine high-resolution crystal structures combined with small-angle X-ray scattering and in vivo biochemical crosslinking, to elucidate a structural and thermodynamic mechanism for the controlled synthesis of the bacterial flagellar motor, a biological motor consisting of hundreds of subunits that can rotate at over 1300 Hz. The mechanism describes how a structural template can trigger and guide the polymerisation of subunits via a domain-swap mechanism during assembly. Here we describe our efforts to replicate this process in vitro by replacing the natural scaffold with synthetic scaffolds constructed from DNA origami. By observing the kinetics of artificial synthesis, we can probe fundamental questions about supramolecular protein complex assembly and their dynamics.

P17: Visualising ocular lens function and dysfunction with protein imaging mass spectrometry

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The lens is a unique structure in the eye that focusses light onto the retina in order to form a sharp image, and has several adaptations to do this effectively. It lacks a blood supply, has an ordered cellular structure, and loses light scattering cellular organelles as lens fibre cells mature. It also expresses crystallin proteins in high abundance in specific regions of the lens to create a gradient in refractive index that facilitates high visual acuity over several decades of life. The primary lens pathology, age-related nuclear (ARN) cataract, is thought to be due to protein cross-linking and aggregations which form light scattering opacities in the lens. Aberrant protein post-translational modification and antioxidant deficiencies are thought to contribute to the formation of this prevalent form of blindness. Therefore, mapping the spatial distributions of proteins and their modified forms throughout the lens will suggest which modifications are related to cataract formation.

An antibody-based labelling strategy is unable to achieve this due to the high likelihood of antibody cross-reactivity with multiple modified forms of a single protein. However, MALDI imaging mass spectrometry, which simultaneously detects multiple protein isoforms separated by their mass, can be used to address this. MALDI imaging was utilised to map proteomic changes that take place in normal human lens aging, in cataract formation, and in a laboratory model of ARN cataract. Human lenses were removed from the ocular globe and frozen immediately, while bovine lenses were removed from the ocular globe and subjected to hyperbaric oxygen treatment to simulate ARN cataract before freezing. Cryosections from human or bovine lenses were collected on MALDI targets and matrix (sinapinic acid) applied in a thin even coat. MALDI imaging data sets were collected at 150-250□m spatial resolution using a MALDI-TOF mass spectrometer.

In the human lens, multiple C-terminally truncated alpha crystallin fragments were detected, and their abundance increased with age specifically in the lens nucleus. Phosphorylated forms of alpha crystallin were most abundant in lens cortical regions. A novel amino acid substitution in the alpha-A crystallin protein was detected in the cataractous human lens. In the aging bovine lens model, putative glutathionylation of beta and gamma crystallins was detected specifically in cortical regions. The implications of these observations for cataract formation will be discussed.

P18: The structure and function of a bacterial sialic acid transporter

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Sialic acids comprise a varied group of nine-carbon amino sugars widely distributed among mammals and higher metazoans. Commensal and pathogenic bacteria that colonise heavily sialiated niches (e.g. the mammalian respiratory tract and gut) can scavenge sialic acids from their surrounding environment. Scavenged sialic acid is used as a carbon, nitrogen and energy source, or to evade the host immune response by decorating their outer surfaces in sialic acid. Bacterial sialic acid membrane protein transport systems have been identified that belong to the tripartite ATP-independent periplasmic transporters, ATP-binding cassette, major facilitator superfamily and sodium solute symporter transport systems. Here we report the 1.95 Å resolution crystal structure of a specific sialic acid sodium solute symporter, SiaT, in its outward-open conformation. The structure of SiaT was determined in complex with sodium and sialic acid bound, providing insight into how this transporter mediates the movement of sialic acid across the membrane.

P19: Using engineered thermostabilized G protein-coupled receptors for interrogation of ligand selectivity and drug screening using NMR

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G protein-coupled receptors (GPCRs) are the largest class of drug targets, yet the majority remain undrugged, often despite clear linkage to disease. A lack of structural knowledge about how ligands engage GPCRs to modulate complex signalling events has hindered compound optimisation in drug discovery. While recent GPCR crystal structures give us snapshots of how ligands interact with GPCRs more recent NMR work suggests that GPCRs exist in an ensemble of states, with different ligands shifting this equilibrium in different ways upon binding, to influence signaling. This ensemble of states correlates with GPCRs stimulating diverse signaling outputs depending on the bound ligand and the cellular environment. Importantly, this diverse signalling has implications for drug discovery, where particular pathways activated by a receptor may be beneficial, whereas others may cause detrimental side effects. NMR studies to understand ligand mediated GPCR structural diversity requires thermostabilized receptors. Our group has evolved ultra-stable prototype GPCRs using Cellular High-throughput Encapsulation, Solubilization and Screening (CHESS) (1). These receptors demonstrate long-term stability in detergent solutions or lipid nanodiscs at high temperatures and are hence suitable for NMR studies. While all of the stabilized receptors bind ligand most do not signal, however we have also been able to develop signaling competent receptors that respond to agonists in similar manner to wild-type receptors in cell-based assays. We are studying these active and inactive receptors using both ligand-observed NMR methods and isotopic labelling experiments to study receptor dynamics. Because GPCRs are the largest, yet underexploited class of drug targets, this work has broad implications for studying GPCR-ligand interactions and for drug discovery and optimization.

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P20: Dual activity of cytochrome P450c17 - regulation of cortisols and androgens

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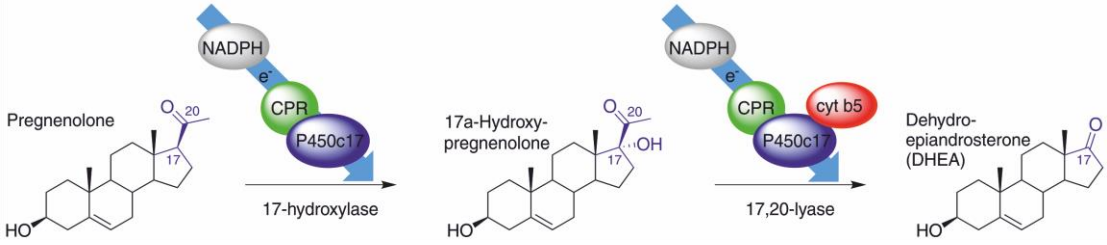

Steroid hormone biosynthesis requires spatial and temporal control of a pathway that begins with cholesterol and directs steroid precursors towards three classes of hormones. These regulate and control salt balance (mineralocorticoids), sugar metabolism (glucocorticoids) and reproduction (androgens). In the case of androgens, cytochrome P450 17α-hydroxylase, 17,20-lyase (P450c17) is the key enzyme. However, P450c17 is a multi-functional P450, so, it synthesises precursors for two of the hormone classes; i.e. both cortisols and androgens. The regulation of this dual activity has been the focus of research studies over many decades and remains unresolved. Functionally, it is known that each of these reactions of P450c17 require electrons transferred by the electron donor cytochrome P450 oxidoreductase (CPR). The first reaction, the 17α-hydroxylation of pregnenolone, occurs in all cells where P450c17 is expressed. Remarkably, the second reaction, namely the 17,20-lyase activity, only occurs for androgen biosynthesis. The specificity of the second reaction is due to a non-redox 'allosteric' interaction with the haem-protein cytochrome b5. Surprisingly, cytochrome b5 and cytochrome P450 oxidoreductase have overlapping binding sites on the surface of the P450c17 enzyme. This poses several questions: How does cytochrome b5 and cytochrome P450 oxidoreductase interact with P450c17 - structurally, functionally and physiologically? Mechanistically, how does the allosteric interaction with cytochrome b5 specifically regulate the P450c17 activity? Finally, how does the **one** enzyme function to achieve the synthesis of two essential classes of steroid hormones, *in vivo*?

We present our data that uses FRET in live cells, biophysical measurements (direct electrochemistry and a quartz crystal microbalance) and molecular modelling that predicts that the P450c17 forms a stable homodimer structure,[1,2] allowing the CPR and cytochrome b5 proteins to form a tetrameric 'functional complex'[3]. This model is fully consistent with extensive experimental data published over the last two decades and could resolve the conundrum as to how P450c17 interacts with two proteins using the same interaction site.

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P21: Structure-function analysis of the antifungal target sterol 14α-demethylase

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Sterol 14α-demethylase (Erg11p, CYP51) in fungi is a bitopic, membrane monospanning cytochrome P450 protein in the CYP51 class that is targeted by the azole drugs and agrochemicals. It is therefore important to understand how the demethylase interacts with the membrane, binds its substrate, product and inhibitors, and how the structure and function of the enzyme is affected by mutations that confer azole resistance. Until recently models of fungal Erg11ps were based on the crystal structures of CYP51s without the N-terminal transmembrane domain. We have used a yeast expression system to obtain high-resolution (1.9 - 2.9 Å resolution) X-ray crystal structures for full-length, C-terminal hexahistidine-tagged, *Saccharomyces cerevisiae* lanosterol 14α-demethylase (ScErg11p) with, and without, substrates or inhibitors bound and for the homologous enzymes from the fungal pathogens *Candida glabrata* and *Candida albicans* in complex with itraconazole. Structures containing single mutations reported to confer azole resistance have also been obtained.

The crystal structures reveal two N-terminal helices oriented at ~60° to each other which orient the partially membrane embedded and conformationally-stable catalytic domain relative to the lipid bilayer. The ScErg11p structures showed lanosterol in the active site, a substrate entry channel linked to the lipid bilayer, and a putative product exit channel. The structures indicate how azole antifungals block catalysis. The Y132F mutation in *C. albicans* Erg11p confers azole resistance on clinical isolates. Yeast cells with the corresponding mutation (Y140F) introduced into ScErg11p became resistant to short-tailed but not long-tailed triazoles. The crystal structure of ScErg11p Y140F showed modified binding of the short-tailed azoles fluconazole and voriconazole but normal binding of the long-tailed itraconazole. The crystal structures of wild type and mutant ScErg11p in complex with azole ligands together with the crystal structures for the lanosterol 14α-demethylases of leading fungal pathogens are facilitating drug design that will more effectively target fungal CYP51s.

P22: Energetic and structural factors controlling membrane association and activity of wild-type and oncogenic H1047R PI3Kα

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Phosphoinositide 3-kinase (PI3Kα) is a novel cancer therapeutic target, which phosphorylates phosphatidylinositol-2-phosphate (PIP_2) to form PIP_3 at the cell membrane surface. Oncogenic mutants of PI3K have altered membrane-binding properties and activities. We have built a model cell membrane and carried out molecular dynamics (MD) simulations of wild-type PI3Kα and the two most common oncogenic mutants, E545K and H1047R. These show that compared to wild-type, H1047R PI3Kα has an increased affinity for the membrane, to which it binds in a catalytically competent orientation independently of its cellular partners (growth factor receptor tyrosine kinases and *Ras* proteins), whereas E545K failed to interact with the membrane. Comparison with simulations using a single-component lipid membrane show that lipid composition is an important factor in PI3Kα membrane targeting. Together, our results point to a possible mechanism by which the H1047R mutation increases PI3Kα activity, which may ultimately suggest ways in which this variant could be targeted while avoiding affecting wild-type PI3Kα.

P23: Exploring the structure and dynamics of phosphoinositide kinases and their role in disease

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Lipid phosphoinositides are essential regulators of many cellular processes, including growth, proliferation, membrane trafficking, and cytokinesis. The generation of lipid phosphoinositides through the action of lipid kinases and phosphatases must be tightly regulated. The misregulation of these enzymes are frequently involved in numerous human diseases, including cancer, viral infection, and inflammation. I will discuss our work examining the regulation of phosphatidylinositol 4 kinases (PI4K) and phosphoinositide 3-kinases (PI3K), and the molecular basis of how they are regulated by both host proteins and viral proteins in disease. A specific focus will be on our synergy of X-ray crystallography, Hydrogen deuterium exchange mass spectrometry, and functional biochemical assays to probe enzyme structure, dynamics, and function. These enzymes all act on membrane surfaces, and I will also focus on our development of novel biophysical tools to examine membrane-signalling complexes in their native lipid environment, and how disease linked mutations change interactions with membrane surfaces

Our research has specifically focused on examining the PI4K isoform PI4KIIIβ and the class IA family of PI3Ks. For the PI4KIIIβ enzyme we will describe our HDX-MS approach to crystallize the protein complex of PI4KIIIβ with the GTPase Rab11 (Burke et al. Science 2014, Fowler et al. Protein Science 2016), as well as how HDX-MS was used to characterize the complex of PI4KIIIβ with an ACBD3-viral protein complex that mediates viral infection (McPhail et al. Structure 2017). For the class IA PI3Ks we will describe our work examining primary immunodeficiency mutants and how HDX-MS was used to define the molecular basis for how these mutants mediate disease (Dornan et al. PNAS 2017). Finally, we will discuss our unpublished work using HDX-MS to characterize the membrane signaling complex of PI3K with the potent oncogene Ras on membranes, and how this approach has provided unique insight into isoform-specific signaling of the class IA PI3Ks.

This work is supported by grants from the Canadian Institute of Health Research (CIHR), and the National Science and Engineering Research Council (NSERC).

P24: Apoptosis Signal-regulating Kinases–linking redox stress to MAPK signalling

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Apoptosis signal-regulating kinases (ASK1-3) are apical kinases of the p38 and JNK stress-activated MAP kinase pathways. They are activated by diverse stress stimuli, including reactive oxygen species, cytokines, and osmotic stress. ASK proteins are relevant to the pathology of both cancer and neurological diseases and have been extensively studied in cells, but a molecular understanding of how they are regulated remains obscure. We are using a variety of structural and biochemical methods to address this disparity and have recently solved the first structures of ASK regulatory regions. Our studies of the central regulatory region of ASK1 suggests that it plays two roles: acting as a scaffold that primes a key ASK1 substrate for phosphorylation; and as a physical tether to facilitate redox-regulation of ASK1 kinase activity. We have also solved the structure of the C-terminal domain of ASK3, which provides a model for oligomerisation of ASK-type proteins into their active signalling complex, the 'ASK-signalosome'. Together, these studies allow us to propose a revised model for regulation of ASK-type kinases, and a platform for future studies of stress-activated signalling by the ASK-signalosome.

P25: Decoding the functions of kinases and phosphatases with bioinformatics - from a billion years of evolution to modern cancer genome sequencing.

Gerard Manning, Mark Jinan Chen, Jacob Rinaldi, Nick Lounsbury, Ryan Hartmaier, Sally Trabucco, Ethan Sokol

The many functions of protein kinases and phosphatases have evolved and been fine tuned over millions of years of evolution. Comparative genomics now allows us to track the sequence evolution of these proteins and to better understand many aspects of their functions, in any organism. I will present an evolutionary vista of both the kinome and phosphatome, and demonstrate both synergy with experimental understanding, as well as challenges that have not been experimentally addressed. I will also show very recent analysis of short-range evolution within human tumors, using a set of over 100,000 tumor genomic profiles to reveal a restricted set of patterns of MAPK activation across different tumor types.

P26: Protein engineering of next generation monobodies

Peter Chandler[1], James Scott Green[1], Benjamin T. Porebski[2], Paul J. Conroy[1], Nyssa Drinkwater[3], Peter Schofield[4,5], Rodrigo Vazquez-Lombardi[4,5], David E. Hoke[1], Daniel Christ[4,5], Sheena McGowan[3], Ashley M. Buckle[1]

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The favorable biophysical attributes of non-antibody scaffolds make them attractive alternatives to monoclonal antibodies. However, due to the well-known stability-function trade-off, these gains tend to be marginal after functional selection. A notable example is the fibronectin type III (FN3) domain which has been evolved to bind several targets of therapeutic interest with high affinity. We have recently designed a new ultra-stable FN3 scaffold which offers exceptional robustness for engineering binding functionality using both rational and directed evolution. I will discuss our recent progress demonstrating the development of high affinity binders to receptors of therapeutic interest.

P27: Structural and functional innovations in the real-time evolution of new (□□)$_8$ barrel enzymes

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New genes can arise by duplication and divergence, but there is a fundamental gap in our understanding of the relationship between these genes, the evolving proteins they encode, and the fitness of the organism. We have used crystallography, NMR dynamics, kinetics and mass spectrometry to explain all of the molecular innovations that arose during a previous real-time evolution experiment[1]. In this experiment, the $(\beta\alpha)_8$ barrel enzyme HisA was under selection for two functions (HisA and TrpF), resulting in duplication and divergence of the *hisA* gene to encode TrpF specialists, HisA specialists, and bi-functional generalists. We discovered that selection affected enzyme structure and dynamics, and thus substrate preference, simultaneously and sequentially. Bi-functionality is associated with two distinct sets of loop conformations, each essential for one function. We observed two mechanisms for functional specialization: structural stabilization of each loop conformation; and substrate-specific adaptation of the active site. Intracellular enzyme performance, calculated as the product of catalytic efficiency and relative expression level, was not linearly related to fitness. Instead we observed thresholds for each activity, above which further improvements in catalytic efficiency had little, if any, effect on growth rate. Our data thus refute a model for 'diminishing returns' in enzyme evolution[2]. Overall, we have shown how beneficial substitutions selected during real-time evolution can lead to manifold changes in enzyme function and bacterial fitness. This work emphasizes the speed at which adaptive evolution can yield enzymes with activities that are sufficiently high that they no longer limit the growth of their host organism, and it confirms the $(\beta\alpha)_8$ barrel as an inherently evolvable protein scaffold.

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2. Tokuriki, N., *et al.* (2012). *Diminishing returns and tradeoffs constrain the laboratory evolution of an enzyme.* Nature Communications, 3, 1257.

P28: Protein superglue from bacteria

Squire, C.J.[1,2], Yosaatmadja, Y.[1], Young, P.G.[1,2], Baker, E.N.[1,2], Harris, P.W.R.[1,2], Leung, I.K.H.[3]

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Elongated bacterial surface proteins require strong and stable structures as they are subjected to harsh environmental conditions. In some of these proteins, stability is achieved though covalent intramolecular crosslinks including disulphide bonds, isopeptide bonds, thioester bonds, and ester bonds, which we have most recently discovered within the repeat domains of a *Clostridium perfringens* adhesin protein.[1] The ester bond crosslinks form spontaneously using an enzyme-like mechanism (serine protease) and provide demonstrable thermal, proteolytic, and tensile stability to the single-molecule-wide protein. We have now engineered the natural domains from this protein and others to produce a molecular superglue – a means to ligate any pair of proteins together.

This new protein ligation system (E-Ligase/RE-Ligase) while similar in principle to the earlier SpyTag/SpyCatcher system based on isopeptide bonds, is inherently more flexible and provides a toolkit of orthogonal building blocks. The power of orthogonal design is obvious when we consider the examples of protein assemblies and nanomaterials produced by a pair of isopeptide tags – we anticipate a massive increase in complexity will be achieved through protein engineering with our superglue tools.

1. Kwon, H., Squire, C.J., Young, P.G. and Baker, E.N. (2014). *Autocatalytically generated Thr-Gln ester bond cross-links stabilize the repetitive Ig-domain shaft of a bacterial cell surface adhesin.* Proc Natl Acad Sci USA. 111: 1367-72.
2. Young, P.G., Yosaatmadja, Y., Harris, P.W., Leung, I.K., Baker, E.N. and Squire, C.J. (2017). *Harnessing ester bond chemnistry for protein ligation.* Chem Commun (Camb). 53: 1502-05.

P29: The structure and function of KstR, the major regulator of cholesterol catabolism in Mycobacterium tuberculosis

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Cholesterol can be a major carbon source for Mycobacterium tuberculosis (M.tb) during infection, both at an early stage in the macrophage phagosome, and later within the necrotic granuloma. KstR is a highly conserved TetR family transcriptional repressor that regulates a large set of genes responsible for cholesterol catabolism. Many genes in this regulon are either induced during infection or are essential for bacterial survival in vivo. Two ligands have been identified for KstR, both of which are CoA thioester cholesterol metabolites with four intact steroid rings that strongly inhibit KstR-DNA binding, and the crystal structures of the ligand-free, ligand-bound and DNA-bound forms of KstR have been determined.

Structures of KstR□-ligand complexes show that the DNA-binding domain is positioned unfavorably for DNA binding. Comparison of ligand-bound and ligand-free structures identifies residues involved in ligand specificity and reveals a distinctive mechanism by which the ligand-induced conformational change mediates DNA release. The structure of the KstR-DNA complex suggests that a bend in the target DNA is structurally required for a snug fit with the two DNA-binding domains of the KstR dimer. Furthermore, data from molecular dynamics (MD) simulations strongly suggests that this signature DNA deformation is encoded in the nucleotide sequence.

As KstR controls the expression of a metabolic pathway that is essential to mycobacterial pathogenesis, it may present a novel opportunity for the development of new anti-TB therapeutics. It is possible that gratuitous inducers of KstR may be able to chemically reproduce the phenotype of the KstR knockout and prevent M.tb from growing in vivo. Conversely, compounds that lock KstR in a DNA-bound form and prevent it from responding to its natural ligands may be expected to prevent M.tb from regulating cholesterol breakdown. Hence, small molecule ligands for KstR have been identified that are chemically distinct from the natural steroid metabolites using a fragment-screening approach.

P30: Targeting NDH-2, an essential bacterial respiratory enzyme for development of novel antibiotics.

Nakatani, Y.[1]

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Tuberculosis (TB) remains one of deadliest diseases in the world, counting 10.4 million incidences and 1.8 million deaths in 2015. WHO has set the “End TB Strategy” to end the TB epidemic by 2030. This involves improving a rapid diagnosis and treatment regimen in the Tb burden countries. Despite a large international effort, *Mycobacterium tuberculosis*, a causative organism of tuberculosis has developed multidrug resistance to the first line anti-TB drugs (480,000 multidrug resistant cases in 2015), and continues to develop resistance to the second line drugs (extensively drug-resistant). To combat against drug resistant TB strains efficiently, it is urgently required to develop new anti-TB drugs with novel modes of action.

Our team focuses on developing inhibitors that target energy generation in *M. tuberculosis*. In particular we target the type II NADH:quinone oxidoreductase (NDH-2), “a validated drug-target” of the *M. tuberculosis* electron transport chain (ETC) components. NDH-2 is a 40–70kDa single-subunit monotopic membrane protein that catalyses the oxidation of NADH and reduction of quinone molecules through the cofactor FAD or FMN. This enzyme plays a central role for both transferring electrons into ETC for generation of ATP and maintaining a cellular NAD^+/NADH balance. In my talk, our research efforts to identify and develop a specific NDH-2 inhibitor in the last five years will be presented. These include determination of the first crystal structure of the bacterial NDH-2, elucidation of the NADH:quinone oxidoreduction catalytic mechanism, identification of 2-heptyl-4-quinolinol 1-oxide (HQNO), a specific inhibitor that targets the quinone binding site of NDH-2, and finally the NDH-2-HQNO complex structure. I also briefly introduce our current research that elucidates the physiological role of NDH-2 in cellular metabolism and virulence in pathogenic bacteria.

P31: Targeting isocitrate lyase for the treatment of latent tuberculosis

Bhusal, R.P.[1], Bashiri, G.[2], Kwai, B.X.C.[1], Patel, K.[1], Reynisson, J.[1], Sperry, J.[1], Leung, I.K.H.[1]

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Tuberculosis (TB) is an infectious disease that is caused by *Mycobacterium tuberculosis*[1]. TB has a long latency period; once a human is infected with *M. tuberculosis*, the bacteria may stay inactive within macrophages for many years leading to a syndrome that is known as latent TB. As *M. tuberculosis* can only spread from those who have developed active pulmonary TB, treatment of latent TB infection for high risk individuals is a viable strategy to control the disease. The enzymes isocitrate lyase (ICL) isoforms 1 and 2 play essential roles in the survival of *M. tuberculosis* in the latent phase[2,3]. ICLs are not present in humans and are therefore promising potential therapeutic targets for the development of new anti-TB agents. In this talk, we describe our use of a combined structural biology, molecular biology, computational chemistry and biophysical approach to obtain structural and mechanistic understandings of the *M. tuberculosis* ICL enzymes. Our work may pave way for the development of new therapeutic agents against TB.

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1. Muñoz-Elías, E.J., McKinney, J.D. (2005). *Mycobacterium tuberculosis isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence*. Nature Medicine. 11: 638–644.
2. Bhusal, R.P., Bashiri, G., Kwai, B.X.C., Sperry, J., Leung, I.K.H. (2017). *Targeting isocitrate lyase for the treatment of latent tuberculosis*. Drug Discovery Today. 22: 1008–1016.

P32: Molecular insights into cofactor F_{420} biosynthesis uncover a revised pathway

Ghader Bashiri[1], Ehab N. M. Jirgis[1], Blair Ney[2], Chris Greening[2], Colin Jackson[2], Edward N. Baker[1]

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F_{420} is low-potential redox coenzyme that functions as an electron carrier in hydride transfer reactions. F_{420} was first identified in methanogens in 1972 and since then has been shown to play an essential role in the metabolism of a wide range of microorganisms. F_{420} is best known for its role in methanogenesis, although it also transforms a wide range of substrates in aerobic actinobacteria, *e.g.* mycobacteria and streptomycetes. While structurally similar to riboflavin, F_{420} shows distinctive and biologically useful electrochemical properties that enhances the metabolic flexibility of microorganisms by catalyzing a wide range of enzymatic redox reactions.

A pathway has been proposed for F_{420} biosynthesis in Archaea and bacteria based on the efforts over the last 45 years. In this pathway, F_{420} is formed *via* incorporating metabolites from the flavin biosynthesis and lactate metabolism. Through accurate identification of the substrate for one of the enzymes in the pathway, we have demonstrated that the F_{420} biosynthesis pathway in both bacteria and Archaea is directly linked to the central carbon metabolism. Based on our targeted metabolomics, biochemical and X-ray crystallographic studies, we propose a revised pathway for F_{420} biosynthesis, which now covers the previously unreported metabolites and enzymatic activities.

P33: Engineering bacterial nitroreductases for biomedical research applications

Dave Ackerley, Victoria University of Wellington, New Zealand

Bacterial nitroreductases are NAD(P)H-dependent oxidoreductases (generally homodimeric and FMN-binding) that can catalyse the 4- or 6-electron reduction of nitro groups on aromatic rings. This results in a profound electronic shift that can dramatically alter the properties of the molecule as a whole, e.g. activating latent cytotoxins, or detoxifying certain pollutants or antibiotics. We have exploited these properties and the characteristic promiscuity of these enzymes to develop useful tools for biomedical research and therapy, in particular the anticancer strategy gene-directed enzyme prodrug therapy and targeted cellular ablation in zebrafish models of degenerative disease. We have used directed evolution to improve desirable activities and are also investigating the use of dual positive and negative selection strategies to tailor reaction specificity and to better understand how the evolution of promiscuous enzymes is modulated by *in vivo* constraints. A further application of our positive selection capabilities has been the recovery of novel nitroreductases from collections of uncharacterised environmental DNA.

Summary of Abstracts for the Poster Session Template

No.	Title	Presenter	Institutions
P34	Finding an Achilles Heel to *Phytophthora agathidicida*	L.B. Armstrong	University of Otago, NEW ZEALAND
P35	Structural characterisation of NlpC/P60 proteins from Trichomonas *vaginalis*	M.J. Barnett	University of Auckland, NEW ZEALAND
P36	Re-targeting a bacterial assassin: Engineering PlyC specificity using directed evolution	Sebastian S. Broendum	Monash University, AUSTRALIA
P37	RHS proteins for pathogenicity and intercellular competition	J.N. Busby	University of Auckland, NEW ZEALAND
P38	Methanogen Non-ribosomal Peptide Synthetases and their role in Methane Mitigation	S. Chunkath	University of Auckland, NEW ZEALAND
P39	Characterizing the constitutive photomorphogenic 1 (COP1) ubiquitin ligase	J.R. Curry	University of Otago, NEW ZEALAND
P40	Building Chains – What features promote processivity?	J.D. Doorman	University of Otago, NEW ZEALAND
P41	Structural Investigations of a Terminal Reductase Domain of a Methanogen Non Ribosomal Peptide Synthetase	Sandesh Deshpande	University of Auckland, NEW ZEALAND
P41	*Pseudomonas* Chemoreceptors: Sequence Identity = Functional Identity?	M.K.G. Ehrhardt	University of Otago, NEW ZEALAND
P43	Quieting the Chatter Behind *Pseudomonas aeruginosa* Infections	M.J. Fairhurst	University of Otago, NEW ZEALAND
P44	Characterisation of the RING E3 ligase RNF125	T.J. Fokkens	University of Otago, NEW ZEALAND
P45	Cryo-electron microscopy investigation of a novel oncolytic picornavirus-receptor complex	Nadishka Jayawardena	University of Otago, NEW ZEALAND
P46	Characterisation of an *Arabidopsis thaliana* plant cysteine dioxygenase	B.K. Hayes	University of Otago, NEW ZEALAND
P47	NMR assignment and dynamics studies of the flexible Menangle virus P protein	N. Herr	University of Auckland, NEW ZEALAND
P48	Fragment-based drug discovery for the transcriptional regulator KstR from *Mycobacterium tuberculosis*	N.A.T. Ho	University of Auckland, NEW ZEALAND
P49	Identification and characterisation of HSP-90 inhibitors	R. Huang	University of Auckland, NEW ZEALAND

P50	Characterization of methanogen nonribosomal peptide assembly lines	Anders Laegaard Jørgensen	University of Auckland, NEW ZEALAND
P51	Irreversible inhibition of FGFR1	Maria Kalyukina	University of Auckland, NEW ZEALAND
P52	Engineering the Substrate Specificity of a PHA Synthase (PhaC)	A. Kane	University of Otago, NEW ZEALAND
P53	Characterisation of the immunomodulatory protein, GIF, encoded by Orf Virus	Theodore W.D. Keats	University of Otago, NEW ZEALAND
P54	Non-ribosomal peptide synthetases from methanogenic archaea	Verne Lee	University of Auckland, NEW ZEALAND
P55	Understanding selectivity in irreversible FGFR4 inhibition	Sean Xiaojing Lin	University of Auckland, NEW ZEALAND
P56	Regulation of E2s: a role for additional ubiquitin binding sites?	A.J. Middleton	University of Otago, NEW ZEALAND
P57	Decoding the Dual-Target Antiactivator QseM	C.R.P. Morris	University of Otago, NEW ZEALAND
P58	Proteomic analysis of 4-phenylbutyrate treated HepG2 cells stably expressing ATP-binding cassette transporter A1 (ABCA1) mutants	J. Munir	University of Otago, NEW ZEALAND
P59	A systematic study to identify peptides involved in the formation of whey protein nanofibrils	Smitha U. Nair	University of Auckland, NEW ZEALAND
P60	Shortening the C-terminus modifies the fitness of three intradiol dioxygenases	Ali Reza Nazmi	Scion, NEW ZEALAND
P61	Understanding the molecular basis of E3-E2 selectivity	K. Nguyen	University of Otago, NEW ZEALAND
P62	RNA ligase from the hyperthermophilic archaeon *Pyrococcus furiosus*	T. Oulavallickal	University of Otago, NEW ZEALAND
P63	Regulation of ubiquitin transfer by Ark2C	A. Paluda	University of Otago, NEW ZEALAND
P64	Computationally exploring the Molecular Dynamics of KstR	A. Razzak	University of Auckland, NEW ZEALAND
P65	Structural and biophysical characterisation of human keratin-associated proteins (KAPs)	Othman Rechiche	University of Auckland, NEW ZEALAND
P66	Expression and purification of a drug target squalene monooxygenase	Alia A. Sagatova	University of Otago, NEW ZEALAND
P67	Tackling Bacterial Resistance to “last-resort” antibiotics through targeting	S.J. Son	University of Auckland, NEW ZEALAND

	MCR-1		
P68	Towards solving the structure of the chromosome condensing protein Lsr2 in complex with DNA	E.L. Summers	University of Waikato, NEW ZEALAND
P69	Structural and biophysical characterisation of the master regulator TRIM28	Y. Sun	University of Auckland, NEW ZEALAND
P70	Correlation of Amino Acid Sequence with Bioactivity using Sheep Cheese Whey Beta-lactoglobulin	Hannah Sunde	University of Otago, NEW ZEALAND
P71	Characterisation of interactions between Trim28 and members of the MAGE protein family	J.R.H. Taka	University of Auckland, NEW ZEALAND
P72	Purification and chromophore composition of an unusual phycoerythrin from New Zealand red alga, *Polysiphonia strictissima*	P.I. Uyseco	University of Otago, NEW ZEALAND
P73	Mammalian Expression of SPRASA (Sperm protein reactive with anti-sperm antibody)	C. Walker	University of Auckland, NEW ZEALAND
P74	Protein Engineering of Diabodies: Developing Tools for Structural Biology	T.J. Weller	Monash University, AUSTRALIA
P75	Insights into apoptosis-signal regulating kinase 1 regulation	Johannes F. Weijman	University of Otago, NEW ZEALAND
P76	Investigating the Dynamic Consequences of Prostate Specific Antigen SNPs	E.C. Wilson	Monash University, AUSTRALIA
P77	The activity of TRAF homo- and heterodimers is regulated by Zinc Finger 1.	J. Zhu	University of Otago, NEW ZEALAND
P78	Investigation the TRIM5α RING and B-box domains in self association in antiretroviral signalling	X.J. Yang	University of Auckland, NEW ZEALAND

Finding an Achilles Heel to *Phytophthora agathidicida*

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Phytophthora cause diseases in a huge range of environmentally and agriculturally important plants, with a worldwide economic impact estimated in the billions of dollars per annum. A species of *Phytophthora* of interest in New Zealand is *P.agathidicida,* which is the causative agent of kauri dieback disease. Kauri are taonga to northern Māori, and an icon for all New Zealanders. Kauri trees are also a keystone species that is absolutely critical for the health and biodiversity of the surrounding ecosystem. Kauri dieback is continuing to spread, and with no known cure, almost all infected trees will die.

Phytophthora produce free-swimming zoospores that are able to chemotaxis towards its next host plant and infect it. *P.agathidicida* exclusively infects kauri trees, indicating there is a special plant-microbe interaction occurring. G-protein coupled receptors (GPCRs) are hypothesised to be responsible for zoospores sensing external plant signals[1]. Activated GPCRs and phospholipid signalling pathways have been shown to play an essential role in *Phytophthora* zoospore development and infection[2].

The aim of this research is to develop urgently needed new tools for reducing the spread of Kauri dieback, and/or protecting iconic individual Kauri trees. By understanding *Phytophthora's* chemotaxis behaviour we believe a new approach to manage kauri dieback can be produced. We are currently looking at screening the chemosensory repertoire of *P.agathidicida* and identifying and characterising the receptors that mediate host recognition. We also screened potential antimicrobial compounds to assess their effect on actively growing mycelial mats, zoospore motility, and zoospore germination. Our results have revealed a number of potential anti-Phytophthora compounds and the results of these screens will be presented.

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Structural characterisation of NlpC/P60 proteins from Trichomonas *vaginalis*

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Trichomonas vaginalis is an obligate parasite, and the causative agent of trichomoniasis. It is the most common non-viral sexually transmitted disease world-wide, with over 260 million cases annually. Treatment options are limited to metronidazole or tinidazole, but is compromised by poor diagnostics, side-effects leading to low compliance, and the emergence of resistant parasitic strains. Infection by T. vaginalis infection is characterised by adherence of the protozoa to vaginal epithelial cells. A process that is inhibited by the natural microflora of the vagina.

The draft genome of *T. vaginalis* showed an expansion in cysteine peptidases with over 460 separate open reading frames encoding peptidases identified. Previous work in our group have identified a set of 9 NlpC/P60 proteins, a super-family of cysteine endopeptidases commonly involved in bacterial cell wall degradation. Co-incubation experiments have demonstrated that overexpressed NlpC genes result in a reduction of bacterial cell counts in culture, providing *T. vaginalis* a competitive advantage.

To investigate the function of the NlpC genes from *T. vaginalis* we have undertaken a structural and biochemical characterisation of 3 NlpC proteins from *T. vaginalis*; NlpC 6, 8, 9. Substrate specificity assays demonstrated NlpC 8 and NlpC 9 were capable of degrading peptidoglycan from both *E. coli* and *B. subtilis.* Unlike previously characterised NlpC 1, 2, and 7, these enzymes digest both pentapeptide and tetrapeptide forms of peptidoglycan stem peptides.

We determined the structures of NlpC 6 and NlpC 9 by X-ray crystallography. Comparison of these structures with the other NlpC proteins identify elements that may play a role in substrate specificity. A co-complex of NlpC 9 with the cysteine protease inhibitor E64 suggests these proteins may be a viable target for future therapeutic molecules. Inhibiting *T. vaginalis* NlpC proteins may remove the advantage it gives the parasite over the microflora of the vagina.

Re-targeting a bacterial assassin: Engineering PlyC specificity using directed evolution

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Resistance to our current antibiotics is reaching crisis levels and there is an urgent need to develop antibacterial agents with novel modes of action. A promising alternative to antibiotics are the naturally occurring endolysin enzymes from bacteriophage. Endolysins cause bacterial lysis by degrading the bacterial peptidoglycan cell wall. Exogenous application of endolysins results in rapid and specific elimination of Gram-positive bacteria making them an excellent alternative and/or adjunct to traditional antibiotics. The streptococcal C1 phage lysin, PlyC, is the most potent endolysin described to date and can rapidly lyse Group A, C and E *Streptococci* [1]. We intend to engineer the specificity of PlyC using a directed evolution approach, to retarget its bacteriolytic activity to other groups of *Streptococci*. We have previously determined the X-ray crystal structure of PlyC, revealing a complicated and unique arrangement of two catalytic domains bound to an octameric cell-wall docking assembly [2]. In this assembly, five residues, previously shown to be important for cell–wall binding, were targeted for site-saturation mutagenesis. This generated five libraries of several hundred clones, which are currently being screened for lytic activity against different bacterial strains. These results will provide several new PlyC mutants that display lytic activity against previously untargeted bacterial strains. In addition, these results will provide an exhaustive mutational analysis of the known cell-wall binding site in PlyC, delivering valuable insight into the interaction between PlyC and its target bacterial cell wall.

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RHS proteins for pathogenicity and intercellular competition

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RHS repeat elements were originally identified in bacteria in 1984[1]. Named rearrangement hot spots, they were initially described as repetitive DNA sequences but are now known to be large protein-encoding genes. Many possible functions have been proposed for these proteins, including carbohydrate binding[2], ligand binding[3], the export of capsular polysaccharides[4], involvement in rRNA synthesis and stationary phase[5], and intercellular competition[6,7].

Recent work has shown that the RHS-repeat-containing BC component of ABC toxin complexes forms a hollow shell that encapsulates a C-terminal toxin domain[8,9]. We have used a combination of bioinformatics, X-ray crystallography, and small-angle X-ray scattering to investigate RHS-repeat-containing proteins in the New Zealand soil bacterium *Yersinia entomophaga*. Several different classes of RHS proteins have been discovered—one that functions as an alternative "C" protein diversify the payloads the ABC complex can deliver, and another that seems to be much more similar to *E. coli* RHS proteins that are thought to be involved in contact-dependent growth inhibition of competing bacteria. The C-terminal domain of this protein was found to inhibit bacterial growth, and this toxicity could be rescued by co-expression of a putative immunity protein.

We propose that RHS repeat proteins have evolved as generic protein delivery vehicles, exemplified by these two distinct roles: one to deliver a toxin into insect cells, allowing the bacteria to colonise the host, and the other to deliver an anti-bacterial toxin to inhibit the growth of competing microbes.

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Methanogen Non-ribosomal Peptide Synthetases and their role in Methane Mitigation.

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Non-ribosomal Peptide Synthetases (NRPSs) are large, modular, multi-domain enzymes that function to synthesize a specific short peptide product in an assembly line fashion. Many NRPSs have been identified and studied in both bacterial and fungal genomes which allow for the production of valuable secondary metabolites. Two ruminant methanogens have recently been shown to possess genes encoding NRPSs, representing the first to be discovered in Archaea. It is hypothesized that the secondary metabolites synthesized by the NRPSs play an important role in methanogen fitness, conferring a survival advantage in the rumen environment. Recent investigation has focused on the mru_0351 NRPS from Methanobrevibacter ruminantium M1 as a part of an overall project aimed at elucidating the secondary metabolites produced by methanogen NRPSs. This involved the optimization of a purification protocol to produce significant amounts of heterologously expressed full-length mru_0351 NRPS, a technically difficult endeavor due to the large size (~470 kDa) of the protein. The purified NRPS was subsequently utilized in a number of biochemical assays to characterize its activity with previously identified candidate substrates. Refinements of the purification protocol, in particular the progressive optimization of a Strep tag chromatography step, resulted in a five-fold increase in the purification yield of mru_0351 NRPS. The presented research contributes to the project's overarching goal of identifying the NRPS's secondary metabolite which may inform the development of novel strategies to target Methanobrevibacter ruminantium M1 and thus reduce methane emissions in ruminant livestock.

Characterizing the constitutive photomorphogenic 1 (COP1) ubiquitin ligase

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Constitutive photomorphogenic 1 (COP1) is a ubiquitin ligase that has been implicated in development of a variety cancers. Our goal is to characterise the relationship between COP1 and its interaction partners—especially substrate adapters, TRIB1 and TRIB2—in order to better understand their role in tumourigenesis.

COP1 consists of a N-terminal RING domain and a C-terminal seven bladed β-propeller fold (WD40 domain), connected by a central coiled-coil. As well as direct substrates, the COP1 WD40 domain binds to the C-terminal tails of TRIB1/2, which recruit further substrates through their pseudokinase domains. We have developed an expression system for the COP1 WD40 domain in an insect cell line from *Spodoptera frugiperda*. Using fluorescence polarization assays we are characterizing the ternary interactions between COP1, TRIB1, and their substrate CEBPα, which is ubiquitylated by COP1 to drive acute myeloid leaukaemia.

We are also investigating the relationship between oligomerization of the COP1 RING domain and its ubiquitin ligase activity, using multiple-angle light scattering, site-directed mutagenesis and ubiquitylation assays. These analyses have revealed that the RING domain of COP1 favours a dimeric state. Together, these experiments will help elucidate the mechanism through which COP1 functions as a ubiquitin ligase for substrates recruited by TRIB1/2, which will guide our understanding of COP1 in the pathogenesis of a variety of cancers.

Building Chains – What features promote processivity?

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Ubiquitylation is a post-translational modification in which the 76-amino acid ubiquitin (Ub) protein is attached to lysine residues on cellular proteins. Ubiquitin modifications can take multiple forms, including the formation of degradative Lys48-linked polyubiquitin chains. Ubiquitin conjugating enzymes (E2s) are a key part of the cascade that attaches Ub to proteins, however regulation of E2 processivity is not yet well understood. The E2 enzyme Ube2K has a ubiquitin binding (UBA) domain which may bind ubiquitin chains, increasing the local concentration of Ube2K to allow for rapid catalysis. We are investigating the ability of the UBA domain to enhance processivity by purifying Ub chains of different lengths and performing ubiquitin transfer assays to measure the rates of ubiquitylation.

To investigate Ub transfer by Ube2K, Lys48 chains have been prepared, separated by MonoS cation exchange chromatography, and purified by size exclusion chromatography. For quantification of ubiquitin transfer, fluorescently labelled Ub will be purified and conjugated to Ube2K. Assays will then be performed using the purified chains of defined length as the substrate. If the UBA domain enhances activity we predict that the longer chains will be more rapidly ubiquitylated by Ube2K. This a good model system for understanding the effect of substrate chain length on E2 processivity.

Structural Investigations of a Terminal Reductase Domain of a Methanogen Non Ribosomal Peptide Synthetase

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Non-Ribosomal Peptide Synthetases (NRPS) are modular enzymes which function as molecular assembly lines to catalyse the synthesis of a wide range of structurally and functionally diverse peptides. A minimum module of an NRPS consists of an adenylation , a thiolation and a condensation domain. The amino acid specific adenylation domain catalyses two reactions: 1) formation of an amino acid adenylate using ATP as a co-substrate and 2) transfer of the adenylated amino acid onto the post-translationally added 4' -phosphopantetheine arm on the adjacent thiolation domain. The condensation domain catalyses the formation of a peptide bond between the amino acids of the two adjacent modules. The resulting peptide is transferred to the thiolation domain of the downstream module. The peptide remains covalently attached to the enzyme until it is released by a termination domain usually present at the C-terminal end of the last module. One type of termination domain is the reductase domain, which catalyses the NAD(P)H dependent reductive release of peptides. Reductase domains show homology to the short chain dehydrogenases/reductases (SDR) family of enzymes and have been reported to catalyse a $2e^-$ or a $4e^-$ reduction of their peptides to produce aldehydes or alcohols respectively.

The current work involves structural characterization of the reductase domain from the recently discovered four module NRPS, mru_0351, from the rumen methanogenic archaea, *Methanobrevibacter ruminantium.* The reductase domain and the preceding PCP domain (351PCPR) of the enzyme was cloned into the pET53-Dest vector and expressed with a N-terminal poly-histidine tag in *Escherichia coli* BL21*. The protein was purified using affinity chromatography followed by size exclusion chromatography to a final yield of around 15 mg/L. Structural and biochemical investigations are being undertaken to characterize the structure and function of this domain.

Pseudomonas Chemoreceptors: Sequence Identity = Functional Identity?

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Bacteria typically live in environments containing nutrients but also potentially hazardous compounds. Therefore, most motile bacteria rely on the process of chemotaxis to move towards favourable and away from unfavourable conditions. During the chemotactic process ligands initially bind to the sensor domain of a bacterial chemoreceptor before an intracellular signal is generated, which eventually leads to a change of swimming behaviour.
Recently, our group characterised the sensor domain of a proline/ γ-amino butyric acid (GABA) binding chemoreceptor (named PscC) from the kiwifruit pathogen *Pseudomonas syringae* pv. *actinidiae* [1]. A BLAST search revealed PscC to be highly similar to a chemoreceptor from *Pseudomonas putida* (McpC, 73 % amino acid identity), involved in cytosine sensing [2]. Intriguingly, despite their different sensory repertoires, the six residues predicted to form the binding site in both sensor domains are completely conserved.

Here we explore the ligand binding repertoires of these sensor domains and aim to identify underlying causes associated with the variation in binding pattern.

High throughput ligand screening identified L-proline and various additional, potential ligands for PscC and McpC. Surprisingly, GABA was found to stabilize PscC, but not McpC, while cytosine was not found to be a ligand for either of the sensor domains. To determine the K_d values for ligand binding isothermal titration calorimetry (ITC) experiments are conducted, while modelling and subsequent mutagenesis of the sensor domains identified amino acid residues associated with high affinity ligand binding.

Overall, the comparison between the two sensor domains has shown that amino acids apart from the predicted binding site critically influence ligand binding, and that the relationship between the sensor domains sequences and ligand binding profiles is more complex than initially assumed.

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Quieting the Chatter Behind *Pseudomonas aeruginosa* Infections

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Pseudomonas aeruginosa is an opportunistic pathogen associated with chronic infections, such as those associated with orthopedic implants and cystic fibrosis. The persistence of *P. aeruginosa* in these infections is enabled by its ability to form biofilms. Biofilms shield the bacterial community in an extracellular polymeric substance, allowing the bacteria to evade host immune responses and antibiotics. As such, biofilms represent a serious health problem in a range of clinical settings.

For *P. aeruginosa*, and many clinically relevant bacterial pathogens, biofilm formation is regulated by a process called quorum sensing (QS). QS is a way for bacteria to communicate via chemical signals and allows them to assess their local population densities. Once the bacteria have reached a critical mass, they activate their arsenal of virulence genes, establish an infection, and/or begin forming biofilms.

We are exploring novel ways to engineer highly active and specific 'quorum quenching' enzymes that irreversibly degrade the signalling molecules used in quorum sensing. Our main focus is *N*-acyl-L-homoserine lactones (AHLs), as they are the most common type of QS molecules used by Gram-negative pathogens. This will allow the patients to be treated more easily with antibiotics, as well as reduce the symptoms of a *P. aeruginosa* infection.

Site-saturation mutagenesis is being used to generate variants of our acylase. These variants are screened using enzymatic assays, *in vitro* biofilm inhibition assays, and a range of high resolution microscopy techniques to evaluate their effect on *P. aeruginosa* biofilms.

Characterisation of the RING E3 ligase RNF125

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The covalent attachment of ubiquitin to substrate proteins is a post-translational modification involved in numerous cellular signalling pathways, most notably the process of protein degradation. Specific transfer of ubiquitin to substrates is assisted by E3 ligases, which lock the ubiquitin carrying E2 conjugating enzyme into a 'closed conformation', where the C-terminal tail of ubiquitin is primed for nucleophilic attack by a substrate lysine residue[1]. The RING E3 ligase RNF125 mediates the formation of degradative signalling Lys-48 linked polyubiquitin chains on specific substrates, such as JAK1 and RIG-1[2,3]. We are interested in the mechanism by which RNF125 generates Lys-48 degradative ubiquitin chains.

Activity assays with truncated RNF125 and Ube2K~Ub conjugate have indicated the RING domain alone is not sufficient for E2 binding, suggesting a role of additional RNF125 domains in stabilizing the RING domain or the E2:E3 complex. Analysis of RNF125 by analytical size exclusion chromatography indicates that it is a monomeric E3 and forms a 1:1 complex with E2 conjugating enzymes, suggesting that regulation of RNF125 activity is independent of dimerisation. We are actively pursuing the crystal structure of RNF125 with a Ube2K~Ub conjugate locked in the closed conformation. Determining the mechanism by which RNF125 operates will lead to a clearer understanding of how RING E3 mediated ubiquitylation is regulated.

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Cryo-electron microscopy investigation of a novel oncolytic picornavirus-receptor complex

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This study is focused on investigating the receptor interaction of Seneca Valley Virus (SVV) using cryo-electron microscopy. SVV is a replication-competent, positive-strand RNA virus belonging to the family *Picornaviridae*. The genome of SVV is packed inside a non-enveloped icosahedral capsid composed of VP1, VP2, VP3 and VP4 structural proteins[1]. SVV is widely recognized as an oncolytic virus that selectively infects cancers with neuroendocrine features, thereby eradicating tumours without causing any off-target effects on normal tissues. The inherent tumour selectivity of SVV has encouraged the investigation of this virus for the systemic treatment of metastatic cancers with neuroendocrine properties such as neuroblastoma and small-cell lung cancer, and has currently progressed to Phase I and Phase II clinical trials[2]. Despite the therapeutic potential of SVV, there is no available literature on receptors mediating the selective tropism of SVV for cancer cells. Recently, in conjunction with our collaborators, we have identified the Anthrax Toxin Receptor 1 (ANTXR1) as a high-affinity cellular receptor for SVV[3]. In the present study, we carried out the binding of ANTXR1 to SVV in vitro, followed by plunge freezing of samples in Leica KF80 for cryo-EM. The samples were visualized on JEM-2200FS cryo-TEM coupled with a DE-20 direct electron detector. The images were processed on RELION for single-particle reconstruction. The 3-D reconstruction from our study is in agreement with the current atomic model of the SVV and depicts the arrangement of VP1 around the five-fold axis and VP2 and VP3 alternating around the three-fold axis. Furthermore, this model demonstrates a radially distributed ANTXR1 density localized around the five-fold symmetry axis of the capsid. Future studies will focus on obtaining a 3-D reconstruction of the capsid-receptor complex at a higher resolution to identify the key residues on the capsid responsible for ANTXR1 binding.

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Characterisation of an *Arabidopsis thaliana* plant cysteine dioxygenase.

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Cysteine dioxygenase (CDO) is a non-heme, mononuclear-iron enzyme which catalyses the addition of molecular oxygen to the thiol group of cysteine residues forming cysteine sulfinic acid. In plants, this dioxidation occurs on N-terminal cysteine residues which promotes N-terminal arginylation and ubiquitin-mediated degradation of target proteins which mediate the response to oxygen depletion. This pathway facilitates both submergence tolerance and seed maturation, contributing to enhanced crop yield.

As mammalian CDO is involved in cysteine catabolism (which prevents the cytotoxic and neurotoxic effects of unregulated free cysteine), determination of its mechanism is clinically relevant. Dual sequence alignment of plant and mammalian CDO suggests that although the same iron-coordinating residues are present in plant CDO, many proposed catalytic amino acids are not. Kinetic- and structural-based characterisation of plant CDO offers a unique perspective on the minimal requirements for CDO activity.

Homology modelling suggests that the central domain of plant CDO is well ordered, adopting a structure similar to mammalian CDO whilst the N- and C- terminal domains are inherently disordered. Bioinformatic analyses show that the N-terminal domain contains a bipartite nuclear localisation signal and *in vitro* experiments indicate that this basic domain also interacts with DNA. Preliminary ^{1}H NMR of the product of plant CDO with free cysteine show spectral features characteristic of cystine, demonstrating that plant CDO is not able to dioxidise free cysteine. In contrast to the lack of activity against free cysteine, colorimetric substrate depletion assays instead show enzymatic activity with short peptide substrates containing an N-terminal cysteine.

Initial characterisation has provided insight into the structure and catalytic function of plant CDO. Further studies are required in order to further understand the CDO mechanism, and to enable manipulation of plant CDO to improve crop yield.

NMR assignment and dynamics studies of the flexible Menangle virus P protein

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Paramyxoviruses cause measles and mumps, the once familiar diseases of early childhood. Other members of this family, such as the bat-borne Menangle virus, present an emerging threat to human health. These viruses possess a unique machinery for replicating and transcribing their genomic RNA, which provides an attractive drug target.

The replicative complex of paramyxoviruses is comprised of three proteins. The nucleocapsid protein (N) packages and organizes the genomic RNA, while the phosphoprotein (P) and the large (L) protein together form the viral RNA-dependent RNA polymerase (RdRp), which replicates and transcribes the genome. L encompasses all enzymatic activities of the RdRp,[1] while P assists the RdRp to engage and move along its protein-RNA template.[2] The ability to act on packaged RNA is the defining feature of the paramyxoviral RdRp, which is without cellular parallel.

P is a functionally active tetramer, which is oligomerized through a centrally located coiled-coil. The region that mediates attachment of the RdRp to its template is C-terminal to the coiled coil. This ~120 amino acid sequence consists of a structured template-binding domain, which is tethered to the coiled coil by an intrinsically disordered linker. The high degree of flexibility in the linker region is believed to be critical for polymerase movement.[3]

Testing this hypothesis requires the detailed investigation of the structure and dynamics of the P protein, which can be achieved using nuclear magnetic resonance (NMR) spectroscopy. Here we present near complete NMR chemical shift assignments for the C-terminal region of the Menangle virus P protein, and report on the dynamic behaviour of this highly flexible protein.

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Fragment-based drug discovery for the transcriptional regulator KstR from *Mycobacterium tuberculosis*

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Mycobacterium tuberculosis (*Mtb*), the causative agent of the human lung tuberculosis (TB), is notorious for its ability to survive and thrive inside the human macrophage. There has been a line of evidence suggesting the critical role of cholesterol metabolism in *Mtb*'s intracellular persistence[1]. The transcriptional regulator KstR regulates a large set of genes encoding the cholesterol degradation pathway[2]. The wide range of KstR's regulatory function and the established importance of its targets indicate this protein may be a potential drug target. Two steroidal CoA thioester intermediates of the cholesterol degradation pathway have been identified as the endogenous inducers of KstR[3]. The protein-ligand interactions have been structurally characterised by X-ray crystallography, which revealed the allosteric mechanism underlying KstR's function.

In this follow-up project, we has probed the potential for the production of compounds targeting KstR via a fragment-based approach. A screen of the 500-fragment Maybridge library has been performed on KstR, using a differential scanning fluorimetry assay as the detection method. The experiment has identified 39 hit fragments, of which two compounds stabilise KstR while the rest show destabilising effects on the protein. The crystal structures of KstR in complex with the two stabilising fragments have shown that the compounds occupy the ligand-binding pocket of KstR, although they do not induce a conformational change as seen with the steroid ligands. In accordance with our efforts in developing new anti-TB drugs of novel mechanisms, these results provide a starting point for further drug-design targeted at KstR.

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Identification and characterisation of HSP-90 inhibitors

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Heat shock protein (HSP)-90 is a molecular chaperone that is essential for the correct folding and functionality of its client proteins in cells[1]. In cancer cells, HSP-90 stabilises cancer-related client proteins that are required for tumour growth and survival. HSP-90 is a current inhibition target for the therapeutic treatments of cancers. Although several compounds have entered clinical trials, some of these compounds are toxic[2]. Therefore there is still a need to further develop inhibitors that target HSP-90.

Virtual screening is a cost-effective and efficient strategy in the identification of structural scaffolds and chemical moieties that are potentially important for binding to a target protein. Biophysical techniques, such as protein nuclear magnetic resonance (NMR) spectroscopy and thermal shift assay, are often applied as complementary methods to verify the hits obtained from virtual screens. In this poster, we describe our work in applying a combined virtual screening, thermal shift assay and protein NMR spectroscopy strategy to identify and validate HSP-90 inhibitors[3].

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Characterization of methanogen nonribosomal peptide assembly lines

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Methanogenic archaea in ruminant livestock are known to be a significant contributor to human-related greenhouse gas emissions, the single largest source being enteric fermentation responsible for an estimated 40% of these emissions. Despite their importance, very little is known about these archaea due to the difficulties and the limited techniques available for culturing them. Recently, two NRPS genes were identified in each of the genomes of the methanogenic archaea, *Methanobrevibacter* sp. SM9 and *Methanobrevibacter ruminantium* M1[1], representing the first archaeal NRPS genes to be identified. Nonribosomal peptide synthetases (NRPS) are macromolecular machines that produce bioactive peptides for which a wide array of important activities have been described. This project focuses on determining the substrates of these NRPS enzymes, using a combination of *in vitro* assays and X-ray crystallography to map out the individual chemical building blocks that the NRPS enzymes use to synthesize their products. The goal of these efforts is to determine the final products of the archaeal NRPS enzymes and characterize its function. Substrate specificity is generally associated with the adenylation domain of the NRPS, which activates the substrate through expenditure of ATP; hence this domain is the primary focus of our project. Experiments have so far yielded substrate candidates for a number of the adenylation domains. Furthermore, we have also determined the structure of one of the adenylation domains with AMP and an amino acid substrate bound in the active site. This represents the first structure of an archaeal NRPS domain, paving the way for a deeper understanding of the similarities and differences between the archaeal NRPS enzymes and their eubacterial and eukaryotic homologues, as well as ultimately elucidating the role of NRPS enzymes in *Methanobrevibacter*.

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Irreversible inhibition of FGFR1

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Lung cancer is one of the leading causes of death worldwide with non-small cell lung cancer (NSCLC) accounting for more than 80% of all cases[1], many cases of which are associated with oncogenic aberrations of fibroblast growth factor receptors (FGFRs) attractive molecular targets for drug development[2.] The FGFR1-4 family proteins are receptor tyrosine kinases for which many small molecule inhibitors have been tested in the clinic – a renewed focus has been on a subset of such molecules, irreversible inhibitors that covalently modify the protein.

A series of irreversible FGFR inhibitors has been tested in cell biology assays at the Auckland Cancer Society Research Centre and were found to be potent inhibitors of FGFR1. We have focused on understanding the structural basis of this inhibition, in particular, how the reactive centre of the inhibitor is presented to the target (Cys488), and also to discover a means to reliably measure the reactivity of the inhibitors against recombinant protein *in vitro*.

We have shown by mass spectrometry the relative reactivity of three series of compounds towards FGFR1 and compared these results to the profiles of published inhibitors FIIN-1 and TAS-120. We hypothesize that inherently lower reactivity correlates with the masking of the acrylamide group in our prodrug compounds compared to the "naked" literature compounds, and that this is probably an advantage in a drug by limiting the potential for toxicity from indiscriminate reactivity. The mass spectrometry assay will also allow us to show in isolated protein experiments, the selectivity of the inhibitors for different members of the FGFR family. We will present preliminary results comparing FGFR1 and FGFR4 that suggest that structural flexibility within inhibitors as well as in the protein P-loop, are critical molecular mechanisms in determining both reactivity and selectivity in FGFR targeting.

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Engineering the Substrate Specificity of a PHA Synthase (PhaC)

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Synthesis of high-grade plastics from petroleum-based feedstocks is a high-cost process resulting in expensive products. Our overall goal is to engineer the pathway of bacterial bio-polyester formation, in order to produce advanced engineering plastics. The introduction of aromatic rings into the main-chain of the polyhydroxyalkanoate (PHA) polymer produced by bacteria may result in a high-grade bioplastic, avoiding the high production cost from complex chemical synthesis. This research aims to create bioplastics from renewable resources, rather than rely on petroleum-based sources.

A key enzyme for this process is the polyhydroxyalkanoate synthase, PhaC. This enzyme is capable of polymerizing activated hydroxybutyrate-CoA monomers. To create a high-grade bioplastic, engineering PhaC to introduce aromatic rings into the polymer is crucial. We have begun by establishing a system that will allow us to use directed evolution to achieve this goal. We have constructed a minimal plasmid for PhaC expression and a second plasmid with the CoA ligase genes required for substrate activation. Life-or-death selection for PhaC variants that can polymerize aromatic substrates will be based upon the toxicity of the un-polymerized hydroxy-acid monomers. We have determined the minimum inhibitory concentrations (MICs) for six of these monomers in *Escherichia coli*. The final stage will be to introduce random mutations into the *phaC* gene by error-prone PCR, and then to select variants that can detoxify the hydroxy-acid monomers by polymerizing them. Progress towards this goal will be reported.

Characterisation of the immunomodulatory protein, GIF, encoded by Orf Virus

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Inflammation serves as the first line of defence of the body against pathogens and injury, however uncontrolled inflammation can lead to serious disease. Currently there are insufficient options for treating inflammation. Parapoxviruses are known to express several immunomodulatory proteins which allow the virus to evade host immune capability. One such protein is Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-2 (IL-2) inhibition factor (GIF), encoded by Orf Virus. GIF binds and inhibits IL-2 and GM-CSF, both of which are important mediators of inflammation. Orf Virus primarily infects sheep and goats, however the disease can transmit to humans as well as common livestock species. With more knowledge of the structural characteristics of GIF it could serve as a template for the development of novel recombinant protein therapies for inflammation.

The crystal structure of GIF in complex with ovine GM-CSF has recently been solved (Felix *et al.*, 2016) and we aim to augment existing knowledge of GIF by characterising its structure with bovine GM-CSF and IL-2. We are currently in the process of producing sufficient quantities of GIF for analysis by X-ray crystallography, SPR and ELISA assays. We have optimised expression and purification of GIF in HEK293E cells. Partial purification has been carried out using immobilized metal affinity chromatography on an FPLC device. Interestingly, instead of a distinct peak, GIF elutes across multiple fractions which we pool together and concentrate. Western blots specific for a hexahistidine tag have shown the purified GIF protein to be 10 kDa larger than theoretical mass (31.6 kDa), consistent with GIF being heavily glycosylated.

Felix J, Kandiah E, De Munck S, Bloch Y, van Zundert GCP, Pauwels K, *et al.* (2016). Structural basis of GM-CSF and IL-2 sequestration by the viral decoy receptor GIF. Nature Communications 7**:** 13228.

Non-ribosomal peptide synthetases from methanogenic archaea

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Non-ribosomal peptide synthetases (NRPSs) are large enzymatic "assembly lines" that synthesise diverse bioactive non-ribosomal peptides, which include many antibiotics and other pharmacologically important molecules. They are modular proteins with each of the repeating modules incorporating a particular amino acid into the peptide being assembled. The modules are further divided into structurally discrete domains that catalyse the chemical reactions necessary for peptide synthesis. Dynamic interactions between these domains play a central role in the function of the enzyme.

While common in fungi and eubacteria, NRPS enzymes are rare in archaea. We are investigating the first NRPS enzymes to be identified in archaea, which are from methanogenic archaea living in the rumens of livestock.[1,2] These archaeal NRPSs are not expressed in culture and nothing is currently known about the non-ribosomal peptides that they produce. We have successfully expressed and purified the large full-length enzymes (up to 475 kDa in size) and a number of smaller constructs from these enzymes using heterologous expression in *Escherichia coli*. We are investigating the structure and function of these enzymes using a variety of structural techniques, biochemical assays and mass spectrometry.

Our investigations to date have revealed that these archaeal NRPS enzymes exhibit unusually relaxed substrate specificity and may each produce a variety of similar non-ribosomal peptides rather than a single specific peptide. Efforts are underway to identify these peptides in order to elucidate the roles that they play in methanogen biology. As non-ribosomal peptides typically confer a competitive advantage to the organisms that produce them, our research may lead to novel targets for inhibiting the growth of the methanogens. This would mitigate enteric methane emissions from livestock, which make up 30-35% of New Zealand's total greenhouse gas emissions and a significant and increasing proportion of global emissions.

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Understanding selectivity in irreversible FGFR4 inhibition

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Aberrant activation of fibroblast growth factor receptors (FGFR1, 2, 3, and 4) drive the development of various malignancies, and small molecule FGFR kinase inhibitors have been developed to target FGFR-associated cancers. Most inhibitors are potent against all four FGFR isoforms (pan-inhibitors), some inhibitors are selective for FGFR1-3, but few are FGFR4-selective. We aim to expand our understanding of what constitutes an FGFR4-selective compound through characterising kinase-inhibitor interactions between FGFR1-4 and several covalent inhibitors at the molecular level. The compounds we are studying include FIIN-1, BLU554, and several novel FGFR4-selective inhibitors developed by our collaborator, Professor Ke Ding, at Jinan University (Guangzhou). FIIN-1 is a pan-FGFR inhibitor which displays no selectivity for FGFR4. By contrast, BLU554 is an FGFR4-selective compound currently in phase I clinical trial (NCT02508467). Our novel series of 2-aminopyrimidine derivatives were developed as novel covalent inhibitors with better FGFR4-selectivity than the parental compound BLU554[1, 2].

To understand the reactivity profile of each compound, we studied the covalent modification of FGFR4 kinase using LC-MS. This confirmed their irreversible inhibition modes and also showed extremely fast reactivity in many cases. We have tried to measure the rate of covalent modification using a time course reaction, but the complete irreversible reaction occurs within seconds for BLU554 and also for our series of novel compounds. In comparison, FIIN-1 while also covalently modifying FGFR4, did so at a much slower (and measurable) rate. LC-MS/MS of FGFR4 tryptic digests will allow us to locate the specific cysteines with the covalent modification, an important consideration for FGFR4 with two active site cysteines as potential reactive centres. Along with X-ray crystallography, the mass spectrometry analysis will provide structural insights into how inhibitors bind to FGFR4, and will aid the rational design of next generation FGFR4-selective inhibitors.

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Regulation of E2s: a role for additional ubiquitin binding sites?

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The post-translational modification of proteins by ubiquitin underlies almost every process in eukaryotic cells. Ubiquitylation can result in different modifications to substrates, including addition of monomeric ubiquitin, or synthesis of polyubiquitin chains with distinct linkage types. Ubiquitin transfer relies on an enzyme cascade that is tightly regulated by a range of signals, including interactions with ubiquitin itself. Thus, ubiquitin can regulate its own transfer to substrates. Specificity of ubiquitylation is provided by E3 ligases, while the ubiquitin conjugating enzymes, or E2s, play a pivotal role in determining the form of ubiquitin modification. Interaction of ubiquitin with E2s is critical, but these contacts have been difficult to study because of their weak nature.

We closely analysed the large number of crystal structures of E2s in complex with ubiquitin. Because protein concentrations are high in crystals, weak interactions tend to be enhanced. Our analysis allowed us to identify well-established ubiquitin-E2 interactions, as well as to discover potential ubiquitin binding sites that have not previously been characterised. These putative interactions may enhance E2 processivity by binding ubiquitin chains, and are likely to regulate assembly of chains of a defined (or homotypic) linkage type.

Decoding the Dual-Target Antiactivator QseM.

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Synthetic biology merges biology and engineering to design and construct synthetic circuits. New bacterial processes can be developed or existing ones adjusted. The inclusion of bacterial chemical cell-cell signalling mechanisms called Quorum Sensing (QS) into synthetic circuits has enabled population-level control. However, biological noise is a significant problem in the creation of more complex circuits as stochastic (seemingly random) or leaky expression of regulatory factors leads to uncontrolled and unpredictable circuit expression. Here we describe a novel dual-target transcriptional antiactivator that has the potential to be used to combat noise and QS crosstalk in synthetic circuits.

The antiactivator QseM tightly represses horizontal transfer of the mobile symbiosis island ICE*Ml*Sym^{R7A} in *Mesorhizobium loti*. QseM binds to and inhibits two transcriptional activators TraR and FseA, independently blocking both quorum sensing and ICE*Ml*Sym^{R7A} transfer. However, the mechanisms by which it inactivates the unrelated TraR and FseA proteins are unknown. We designed and constructed single alanine replacement mutants (alanine scanning) of QseM based on sequence conservation, as well as preliminary structural information and protein-protein interaction experiments. *In vivo* interaction of QseM with TraR and FseA was determined using a *Chromobacterium violacein* CV026 bioassay to detect quorum-sensing signalling molecules and FseA-dependent transcriptional reporter assays respectively. Several substitutions were identified that abolished interaction with both TraR and FseA, but interestingly, one residue was identified that only abolished interaction with FseA, suggesting that QseM interactions with FseA and TraR may be structurally or mechanistically distinct. Further investigation of specific residues, together with the determination of the structure of QseM (see Hall D.A. *et al*., this meeting) will allow us to further pinpoint QseM residues or structural motifs involved in interactions with TraR and FseA, enabling us to exploit the potential of this system for synthetic biology applications.

Proteomic analysis of 4-phenylbutyrate treated HepG2 cells stably expressing ATP-binding cassette transporter A1 (ABCA1) mutants

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The ATP-binding cassette A1 (ABCA1) transporter is a cellular membrane protein that exports cellular cholesterol to form high density lipoprotein (HDL) and protect against cardiovascular disease (CVD). Many mutations in ABCA1 disrupt trafficking to the plasma membrane and reduce its function. We previously showed that the chemical chaperone, 4-phenylbutyrate (4-PBA) can restore membrane localisation and increase the cholesterol efflux function of mutant ABCA1s. The aim of this study was to identify proteins regulated by 4-PBA that restores ABCA1 localisation. Two ABCA1 mutants (p.T1512M and p.N1800H) expressed in stably transfected HepG2 cells were subject to 4-PBA treatment. The cell lysates were prepared from 4-PBA treated and untreated samples and differentially regulated proteins were analysed by mass spectrometric (MS) technique known as sequential window acquisition of all theoretical fragment ion spectra-MS (SWATH-MS). Proteins showing a >2 fold change were subject to bioinformatic analysis using STRING and Panther software packages to investigate the potential association of differentially regulated proteins. The bioinformatic study helped to map the potential functional interaction between ABCA1 and differential dataset of proteins. This analysis identified a number of trafficking proteins that could assist ABCA1 in restoring its plasma membrane localisation. These proteins warrant further characterisation for their potential to rescue ABCA1 localisation.

A systematic study to identify peptides involved in the formation of whey protein nanofibrils

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β-Lactoglobulin, a major bovine milk protein is an extensively studied model for amyloid fibril formation [1,2]. Various mechanisms for fibril assembly have been proposed [1-3], and some of the peptides present in β-Lactoglobulin fibrils have been identified [1,2,4]. In this study, limited proteolysis using trypsin, proteinase K and pepsin, together with MALDI FT-ICR and LC-MS/MS, allowed detailed analysis of β-Lactoglobulin peptides involved in fibril formation. Specific regions of β-Lactoglobulin containing the key peptides for fibrillation were determined by identifying the previously predicted "minimal fibril-forming sequences [3]" in the peptides. We also investigated the role of the protein pH in determining the availability of these sequences for proteolytic cleavage prior to fibril formation.

Limited proteolysis (37 ˚C for 30 min) of whey protein isolate was carried out at pH 7 for trypsin and proteinase K digestion, and at pH 2 for pepsin; followed by hydrolysis at 80 ˚C, pH 2. Fibril formation was confirmed using thioflavin T fluorescence and transmission electron microscopy. Peptides present following hydrolysis were investigated using MALDI FT-ICR to determine the peptide initiating β sheet formation. An LC-MS/MS study on mature fibrils identified all five cysteines in control and pepsin treated sample, while only two cysteines were observed in trypsin and pK treated fibrils. This is hypothesised to be due to the pH of the β-Lactoglobulin protein during proteolysis, playing a major role in determining the availability of peptides for fibrillation due to dimerisation at pH 7. This study has identified peptides crucial for fibril formation, and also provides insights regarding the role of quaternary structure in determining component peptides.

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Shortening the C-terminus modifies the fitness of three intradiol dioxygenases

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Intradiol dioxygenases (EC 1.13.11.1) are bacterial enzymes that catalyze the ring cleavage of catechols that is a central step in the aerobic degradation of aromatic compounds.

Bioinformatics analysis has revealed that some members of this enzyme group have a C-terminus which is 4-5% longer (an additional 13-18 amino acids) than the majority of known sequences.

This longer C-terminus is not highly conserved and appears to be poorly integrated in the available protein crystal structures available from intradiol dioxygenases.Moreover the role of this tail has not been described in the literature.

We selected three enzymes with such an extendeds tail from the model organisms; Burkholderia xenovorans LB400, Pseudomonas putida KT2440 and Acinetobacter baylyi ADP1 to investigate the role of this extended C-terminal tail. We used protein engineering strategies to design variant intradiol dioxygenases by truncating the C-terminus to a size comparable to the shorter versions of the enzyme. These variants and wildtype enzymes were expressed, purified and the specific activity of the truncated enzymes determined on a variety of substrates. The results revealed that truncation of the C-terminus could enhance the activity of the LB400 enzyme by as much as five fold. In contrast, the activity of the intradiol dioxygenases from KT2440 and ADP1 were reduced. We suggest that the difference can be explained by the presence of a greater number of amino acid residues that can contribute to forming stable protein structures in the KT2440 and ADP1 enzymes. In this work we have shown that in some cases C-terminal truncation could provide a useful strategy for increasing intradiol dioxygenase activity for biotechnological applications.

Understanding the molecular basis of E3-E2 selectivity

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Ubiquitylation is a post translational modification that involves the attachment of ubiquitin to substrate proteins. This sophisticated enzyme cascade is accomplished by the activity of an E1 enzyme, an ubiquitin-conjugating E2 enzyme and an E3 ligase. The E3-E2 interaction dictates the ubiquitin code on the substrate protein. To date, determining a comprehensive E3-E2 interaction network remains a challenging question in the ubiquitome field.

Our study focuses on developing a method to systematically screen for E2 partner(s) of several uncharacterised E3 ligases. We are currently focusing on the RING finger protein 121 (RNF121), a membrane associated RING E3 ligase that inhibits the Vascular Endothelial Growth Factor (VEGF) induced angiogenesis in humans. Size exclusion chromatography analysis demonstrated that the RING domain of RNF121 is well folded as a monomer *in vitro.* Biochemical assays that monitor the hydrolysis of the E2-ubiquitin conjugates showed that this E3 ligase interacts with the Ubc13/Uve1a complex. Our next goal is to test the E3 ligase activity of RNF121 with more E2s available in the laboratory. This will predict the ubiquitin code assembled on the target substrate of this E3, and will also give an insight into the molecular mechanism of RNF121. Furthermore, we will apply the same approach to identify the E2 partner(s) of the other uncharacterised E3s.

RNA ligase from the hyperthermophilic archaeon *Pyrococcus furiosus*

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RNA ligases catalyse the ligation of RNA molecules via phosphodiester bonds. While the biological roles of RNA ligases are in processes such as RNA splicing and RNA editing, one of their useful applications is for high-throughput sequencing (HTS) of micro RNA sequences. Micro RNAs are involved in a wide range of cellular processes and aberration in their expression profiles is implicated in various disease states. This highlights the importance of their accurate detection and measurement via HTS. However, micro RNA abundance is often miscalculated as a result of bias caused by stable RNA secondary structures interfering with adapter ligation (1, 2). We are characterising the highly thermostable RNA ligase from *Pyrococcus furiosus* (*Pfu*), because we hypothesise that high temperature ligations could reduce or remove such biases.

The expression and purification of *Pfu* RNA ligase has been optimised. We developed an activity assay for the enzyme and found it to be ATP-dependent. Most importantly, *Pfu* RNA ligase is active at very high temperatures, ranging from 65°C to 90°C. The enzyme also showed the ability to adenylate and deadenylate RNA substrates, similar to the mildly thermostable RNA ligase from *Methanobacterium thermoautotrophicum* (3). Our most recent results on the structure and function of the *Pfu* RNA ligase will be presented. Overall, we are hopeful that the enzyme will find utility in micro RNA HTS protocols.

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Regulation of ubiquitin transfer by Ark2C

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Ubiquitylation is a post-translational modification executed by a cascade of enzymes called E1, E2, and E3. The E3 ligases provide specificity to the cascade and regulation of their activity is critical to the spatial and temporal control of protein ubiquitylation. In recent years it has become apparent that E3 ligases are regulated in different ways[1]. Notably, research in our laboratory revealed that the E3 ligases, Arkadia and Ark2C, bind ubiquitin (Ub^R) and that this ubiquitin molecule regulates activity[2].

Arkadia and Ark2C interact with two different E2s, UbcH5b and Ubc13, which generate distinct signals. UbcH5b mediates synthesis of Lys48-linked degradative poly-ubiquitin chains, while Ubc13 produces Lys63-linked non-degradative chains. Previous structural and biochemical analysis of Ark2C focused on its interaction with UbcH5, and the formation of degradative chains. To understand whether the regulation of Ark2C by Ub^R is conserved for all E2s, we characterised Ark2C:Ubc13 mediated ubiquitylation.

By comparing the activity of wild-type and mutant versions of Ark2C we showed that Ub^R also enhances activity with Ubc13 but other stabilizing residues are less important. Our goal is to visualise the catalytically active complex. So far the Ark2C:Ubc13~Ub:Mms2 complex has been crystallised. This showed that the Ark2C:Ubc13 interface is almost identical to that seen in the Ark2C:UbcH5b complex. To further understand how Ark2C primes ubiquitin for catalysis, additional structural analysis is required. We hypothesise that Ub^R extends the binding surface of Ark2C to stabilize Ub^D. Additionally, mono-ubiquitylated substrate could act as Ub^R and trigger processive ubiquitylation.

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Computationally exploring the Molecular Dynamics of KstR

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KstR is a transcriptional repressor and a member of the TetR family of proteins. It is conserved among mycobacterial species and regulates expression of the enzymes responsible for cholesterol catabolism.[1] Of most interest, KstR is involved in the pathology of Mycobacterium tuberculosis (Mtb), the causative agent of the disease Tuberculosis. Tuberculosis causes more deaths annually than any other bacterial disease.

Mtb is capable of persisting under conditions of extreme metabolic stress, resulting in chronic infections.[2] In its human host, Mtb shows a preference for cholesterol as a carbon source.[1] It has been found that cholesterol metabolites binds to KstR, preventing it from repressing the transcription of the cholesterol catabolism regulon.[3] If Mtb is deprived of its potential to utilise carbon sources such as cholesterol, its ability to form a persistent infection may be hindered.

I have pursued computational Molecular Dynamic simulations of KstR to investigate how the protein functions when in the presence of DNA and ligand, and when free in solution. My experiments focus on quantifying the changing geometry and kinetics of KstR. Consequently they have provided a greater understanding of the protein's interaction with DNA and ligand, while also identifying residues which are potentially critical for its function. Analysis of the experimental material presents information which describes the mechanism of KstR at a molecular level.

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Structural and Biophysical characterization of human Keratin-Associated Proteins (KAPs)

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Keratin-associated proteins (KAPs) were identified 70 years ago in wool follicles. KAPs are encoded by several multi-gene families and are classified into three different groups: ultra-high sulfur (UHS), high sulfur (HS) and high glycine-tyrosine (HGT). KAPs are the major constituent of the matrix between the hair keratin intermediate filaments (IFs), and stabilise hair structure by extensive disulfide bonding. In human, 100 different KAPs are expressed by the hair follicle. These proteins have several distinctive primary structure features, including low sequence complexity and the presence of repeated motifs. Their size varies from 4kDa to 54kDa and and their pI from 4 to 9.
We have expressed and purified a HS KAP (KAP11.1) and a HGT KAP (KAP6.1). The expression and purification of KAPs is challenging because they are cysteine-rich proteins with unusual amino acid compositions. They tend to be insoluble in isolation and are prone to forming aggregates in solution. Therefore, we desiged a method that enables a high yield production of pure, soluble KAPs in a chaotrope- and detergent-free buffer. In order to gain detailed molecular and structural information on KAP11.1 and KAP6.1, we carried out biophysical and structural characterization by Circular Dichroism, SEC-MALLS and SAXS experiments. SAXS experiments showed that KAP11.1 adopts an extended shape in solution and is highly flexibile, whereas KAP6.1 is more compact and present secondary structure features. SEC-MALLS experiments confirmed that KAP11.1 is monomeric in solution, but KAP6.1 shows a concentration-dependent transition from monomer to dimer. Another interesting feature is that both KAP11.1 and KAP6.1 can form hydrogels. This phenomenon depends on different factors: incubation time, protein concentration and temperature. Small volume, highly concentrated sample tend to gelify more readily. The gelification process is also slower when samples are incubated at 4°C in comparison to room temperature. Examples of hair protein and peptide-based hydrogels and their application in severe wound healing cases have been reported in the literature, as well as their commercial derivatives. However, no hydrogel made of a pure KAP has been reported to date.

Expression and purification of a drug target squalene monooxygenase

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Heart disease and cancer are leading causes of death. Fungal infections afflict billions and these "hidden killers" are lethal for almost 1.4 million people per annum, especially those with co-morbidities or immune deficiencies. The membrane protein squalene monooxygenase (Erg1) is a key enzyme in all three diseases. It catalyses a committed early step in cholesterol synthesis of mammals and ergosterol synthesis of fungi and is a validated drug target. Inhibition of fungal Erg1 by terbinafine is widely used to treat dermatophytoses but its narrow spectrum of action precludes treatment of lethal systemic infections by other fungi. Human Erg1 (HsErg1) is a validated target for inhibitors to succeed side-effect prone cholesterol-lowering statins and sterol-reducing anticancer drugs. In the absence of published crystal structures for Erg1, homology models using the structure of the soluble *Pseudomonas fluorescens* p-hydroxybenzoate hydroxylase provide limited insight.

We have used our patented yeast expression system to express Erg1 from humans, the model yeast *Saccharomyces cerevisiae* and the fungal pathogens *Candida glabrata* and *Candida albicans*. Codon optimised *CaERG1* and *HsERG1* genes incorporating hexahistidine affinity tags were commercially synthesised. Functional expression of ScErg1, CgErg1 and HsErg1 has been confirmed using microdilution drug susceptibility assays with terbinafine. Only CaErg1 expression was insufficient to confer resistance. The full-length hexahistidine-tagged ScErg1 has been solubilized using the detergent n-Octyl-β-D-Glucopyranoside and purified by Ni-NTA affinity and size exclusion chromatography. The purification, biochemical characterization and X-ray crystallography of the full-length Erg1 enzymes will allow the identification of membrane interactions required for metabolic regulation and drug binding sites needed for structure-based drug design. The project will yield new knowledge of membrane protein structure, advance target-based drug discovery and ultimately provide new therapeutic options for patients with heart disease, cancer, and for superficial and potentially lethal disseminated fungal infections.

Tackling Bacterial Resistance to "last-resort" antibiotics through targeting MCR-1

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In November 2015, a new antibiotic resistance gene was discovered in China that can be easily transferred and copied between different bacterial strains. The gene, *MCR-1*, makes common bacteria highly resistant to the "last-resort" class of antibiotics called polymyxins.

The MCR-1 gene encodes a 541 amino acid membrane protein. Although its exact function is not known, sequence alignment and bioinformatics studies suggest that the MCR-1 protein is homologous to phosphoethanolamine (PEA) transferase. PEA transferase catalyses the addition of PEA moieties to lipid A, which is the lipid component of lipopolysaccharides found on the outer membrane of gram-negative bacteria[1]. Effectively, the modification of membrane lipid shields the bacterium from the binding of the polymyxin antibiotics.

The discovery of this polymyxin-resistant gene that can be easily transferred between different bacterial populations, is a big concern given the emergence of multidrug-resistant (MDR) bacteria, which are resistant to almost all other currently available antibiotics[2]. If these MDR bacteria acquire polymyxin resistance there will be no effective therapies for people who are infected by these MDR superbugs. A priority in antibiotics research is therefore to tackle polymyxin resistance by targeting MCR-1.

In our laboratory, we have successfully expressed and purified the soluble domain of MCR-1$^{(216-541)}$, which was subsequently crystallised and diffracted to ~1.7 Å. This new structure of MCR-1 displays three zinc atoms in or near the putative active site of the protein. Furthermore, by using high throughput virtual screening, we have identified 29 virtual hit compounds. Five of these appear to interact with the protein as observed by differential scanning fluorimetry. Further experiments are in progress to characterise the protein-ligand interactions.

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Towards solving the structure of the chromosome condensing protein Lsr2 in complex with DNA

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Lsr2 is a DNA binding protein that is highly conserved in mycobacteria and related actinomycetes and it is thought to be essential in *Mycobacterium tuberculosis*. Previous studies have shown that Lsr2 is involved in down-regulating a range of genes involved in cell wall synthesis and metabolic functions and it is proposed that it does this by organisation of bacterial chromatin. We solved the structure of the N-terminal dimerisation domain of Lsr2 using crystallographic *ab initio* approaches[1] whereas the C-terminal DNA binding domain structure was solved by others using NMR[2]. Electron microscopy shows that Lsr2 organises DNA into large helical structure involving several strands[1]. Whilst the DNA binding mechanism is modelled based on the NMR structure, the exact mechanism of DNA binding by the entire protein is unknown. Lsr2 contains a long flexible loop between the two domains which may lead to the protein having a large range of movement, allowing it to bind DNA in a dynamic way. The Lsr2 dimer is also capable of forming long oligomers through the interaction of overlapping Nterminal residues and evidence of this has been shown in our crystal structure and using TEM [1].

We wish to solve the structure of Lsr2 bound to DNA. For the purposes of crystallisation of Lsr2 we have engineered the removal of important N-terminal residues involved in oligomersiation to prevent this process occurring in solution. The truncated form of Lsr2 provides a sub-population of purified Lsr2 that is DNA-free and we have utilised this population for binding specific dsDNA oligonucleotides to for crystallisation. To date, we have determined the optimal length of DNA required and have refined the exact combination of nucleotides for ideal protein binding. We have progressed through the crystallisation of Lsr2 bound to multiple oligonucleotides that have yielded poorly diffracting crystals. Currently we are focussing our efforts to improve resolution.

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Structural and biophysical characterisation of the master regulator TRIM28

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TRIM28 (KAP1/TIF1B) has been labelled the 'enigmatic master regulator of the human genome' due to its involvement in a multitude of cellular processes including cell growth and differentiation, pluripotency, transcription repression, DNA repair and tumorigenesis. TRIM28 does not directly bind to DNA, rather it is recruited to the target genes by the Krab-ZFPs (Kruppel associated box-containing zinc finger proteins) - the largest single family of transcription regulators in mammalian cells (Iyengar & Farnham, 2011). Once recruited, TRIM28 acts as a scaffold and recruits effector proteins and protein complexes involved in repressive histone modification and *de novo* DNA methylation, ultimately leading to chromatin condensation and gene silencing. However, the mechanism of TRIM28-mediated silencing and the various protein-protein interactions that are fundamental to this process remain poorly understood.

TRIM28 belongs to the TRIM protein family with over 70 members in humans. They share a conserved N-terminal tripartite motif (TRIM), also known as the RBCC as it consists of a RING domain, one or two B-box domains and an antiparallel coiled-coil (Goldstone et al., 2014). Recent studies have highlighted the importance of TRIM protein self-assembly in their function (Keown, Yang, Douglas, & Goldstone, 2016; Koliopoulos, Esposito, Christodoulou, Taylor, & Rittinger, 2016). We have characterised TRIM28 self-assembly using structural and biophysical techniques and identified the key domain responsible for higher-order assembly. Furthermore we have crystallised and identified the assembly interface. Based on our crystal structure we have designed mutations that blocked self-assembly, and investigated the functional effects of these mutations. Our knowledge in TRIM28 self-assembly will be an important step in delineating TRIM28 function.

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Correlation of Amino Acid Sequence with Bioactivity using Sheep Cheese Whey Beta-lactoglobulin.

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Sheep cheese whey is an underutilised by-product of the cheese making process. Beta-lactoglobulin (-Lg) comprises 50 % of the total whey protein and resists hydrolysis in the gut. Enriched -Lg was obtained by ion exchange chromatography. -Lg was hydrolysed with various novel non-gut proteases and the protein breakdown was analysed using 1D SDS Page. Hydrolysis of bovine -Lg has been shown previously to generate bioactive peptides that have potential for use in health promoting applications. Novelty arises from differences in the amino acid sequence of -Lg and the use of non-gut proteases with different hydrolytic specificities that provides the potential for the generation of novel bioactive peptides from sheep -Lg.

Incubation of ovine -Lg with one of the fungal protease preparations caused extensive hydrolysis of the protein into peptides of 20 residues or smaller. Separation of the hydrolysate by RP-HPLC indicated fractions that had higher antioxidant bioactivity than others. Peptide sequences were obtained by mass spectrometry and bioactive peptide information was obtained from the literature highlighting key sequences. Specific peptide sequences were obtained as synthetic peptides which were analysed for antioxidant and antihypertensive bioactivities. This enabled correlation of bioactivity with specific amino acid sequence.

Synthetic peptides were analysed for free radical scavenging antioxidant capability using an oxygen radical absorbance capacity (ORAC) assay. Bioactivity analysis also included an anti-hypertensive (ACE inhibitor) assay to evaluate the ability of peptides to lower blood pressure. These approaches have identified novel peptide sequences exhibiting bioactivity and contribute further to understanding peptide sequence relationship to bioactivity. There is considerable potential to use -Lg as a natural food additive in health promoting applications.

Characterisation of interactions between Trim28 and members of the MAGE protein family

Taka, J.R.H, Sun, E., Keown, J.R., Goldstone, D.C.

TRIM28 is involved in many cellular processes including transcription regulation, differentiation, development and cancer[1]. As a member of the trim protein family Trim28 shares a conserved N-terminal domain architecture consisting of a RING E3 ubiquitin ligase domain, 2 B-box domains and a coiled-coil region. Members of the MAGE protein family have been shown to modulate Trim28 E3-ubiquitin ligase activity resulting in degradation of the tumour suppressors p53 and AMPK[2]. MAGE proteins are melanoma associated antigens that are restrictively expressed in germline cells but upregulated in a variety of cancers making them a marker of tumorigenesis[3]. To understand how MAGE proteins modulate TRIM28 activity we have attempted to express and purify constructs of Trim28 and MAGE proteins for biophysical and structural analysis.

The Trim28 Ring-Bbox-Coiled-coil (RBCC) construct was expressed and purified and the oligomeric state analysed using SEC-MALLS. Our attempts to purify the MAGE A3 MAGE homology domain (MHD) consistently produced a soluble aggregate, co-eluting with multiple high molecular weight species that are likely cellular chaperones. Optimisation of purification using solubilising and stabilising additives failed to improve solubility. Electron micrographs taken of MAGE A3 MHD-MBP confirmed the protein formed random aggregates rather than ordered assemblies. In a similar manner purification of the MHD from MAGE C2 resulted in protein free of chaperones but still eluted in the void by size-exclusion chromatography.

Further work is required to optimise expression and purification of MAGE proteins for structural and biophysical analysis including co-expression with Trim28 constructs.

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Purification and chromophore composition of an unusual phycoerythrin from New Zealand red alga, *Polysiphonia strictissima*

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Phycoerythrin is a water soluble photosynthetic protein present in cryptomonads, cyanobacteria, and red algae. The relative amounts of its constituent chromophores, phycourobilin and phycoerythrobilin determines phycoerythrin's spectral properties and commercial value. Phycourobilin absorbs light at 495 nm, and phycoerythrobilin at 565 nm. With an absorption at 495 nm, and emission wavelength of 575 nm, phycoerythrin is a complementary dye to other dyes, such as fluorescein. With its absorption coefficient and great Stokes shift, phycourobilin-rich phycoerythrin offers enhanced sensitivity for biomedical assays.

A phycourobilin-rich phycoerythrin was purified from the New Zealand red algae, *Polysiphonia strictissima*, obtained from the Otago Harbour, and characterised. Purification used differential ammonium sulfate precipitation, anion exchange chromatography, and gel filtration chromatography. The subunits were then separated by C_4 reverse phase chromatography and the ratios of chromophores associated with each subunit were then determined by visual absorption spectroscopy. Results show the chromophore composition for each subunit separated. It also suggests that there might be more than one phycoerythrin present in the sample. Peptides containing one chromophore will be generated and characterised by *de novo* sequencing using mass spectrometry to establish at which residue chromophores are covalently attached, and to determine if there is more than one phycoerythrin present.

Phycoerythrin isolated from *P. strictissima* harvested in the summer carries two phycoerythrobilin chromophores on its α-subunit and two phycoerythrobilin and one phycourobilin chromophore on the ß-subunit. Ongoing analysis suggests that during autumn there is a rise in the phycourobilin content of phycoerythrin from *P. strictissima*. Complete analysis of the seasonal variation of this phycoerythrin will determine its potential commercial value, how the seasons affect this, and potentially determining the ideal time for harvest.

Mammalian Expression of SPRASA (Sperm protein reactive with anti-sperm antibody)

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SPRASA (Sperm protein reactive with anti-sperm antibody), with a role in fertility, was independently identified by two groups in the earlier part of this century. This protein has high structural and sequence similarity to lysozyme c and has been classified as a lysozyme-like protein, within the family of c-type lysozymes. Research has identified a role for SPRASA in fertility, in the fertilisation process and SPRASA has also been identified as a cancer testis antigen. However, many aspects of SPRASA's role in biology remains elusive, almost nothing is known about its molecular function.

Despite popular use in recombinant protein expression, bacterial expression of hSPRASA (human SPRASA) resulted in expression within inclusion bodies. SPRASA contains eight cysteines responsible for forming four disulphide bonds which cannot occur in the reducing environment of bacterial cytoplasm. Rapid dilution, on-column refolding, buffer exchange into acidic conditions prior to dilution into low additive refolding conditions were investigated in refolding hSPRASA. These methods largely resulted in the aggregation of hSPRASA suggesting refolding to be more complex than previously thought, despite structural similarities to lysozyme (refolding model). The use of refolding screens did result in the identification of more promising refolding conditions containing low concentration of Arginine, an aggregation suppressor. Further investigation of the promising conditions is required.

While mammalian expression of recombinant proteins is less popular, it was used to investigate soluble hSPRASA expression. Successful expression of hSPRASA in HEK293F cells was confirmed using both anti-SPRASA antibodies in a Western Blot and peptide fingerprinting using mass spectrometry. Therefore, hSPRASA can successfully be expressed in mammalian cells and optimisation of expression would allow for functional studies and x-ray crystallography to be conducted.

Protein Engineering of Diabodies: Developing Tools for Structural Biology

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Diabodies are bivalent and mono or bispecific antibody fragments currently used in diagnostics and as cancer therapeutics.[1] These fragments have two outward facing binding domains in a back-to-back conformation. Structural analysis suggests that diabodies possess conformational flexibility, which may impact on their function and stability.[2] We are using protein engineering and computational approaches in order to explore diabody dynamics with the aim of modulating their conformational properties and thus potential application.

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Insights into apoptosis-signal regulating kinase 1 regulation.

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Apoptosis signal-regulating kinase (ASK) 1 is a mitogen activated protein kinase kinase kinase (MAP3K) that activates the p38 and JNK stress responsive pathways. ASK1 is activated in cells following treatment with hydrogen peroxide (H_2O_2), a major redox-signalling molecule. Whilst cellular conditions that can lead to ASK1 activation are quite well studied, there has been little evaluation of the precise molecular mechanism. ASK1 is a large (~150 kDa) protein with substantial regulatory regions, N-terminal and C-terminal to a central kinase domain. We have used small angle X-ray scattering (SAXS), X-ray crystallography, site-directed mutagenesis and biochemical assays to investigate ASK1 regulation. The N-terminal region of ASK1 contains conserved regions essential for docking of substrate proteins. Additionally, the thioredoxin-binding domain (TBD) within the ASK1 N-terminus can antagonise this docking, facilitating auto-inhibition of kinase activity. Such an auto-scaffolding and redox regulation has been hitherto unclassified within mammalian MAP3Ks. Additionally, we propose a mechanism whereby the small oxidoreductase, Thioredoxin 1, can bind to the TBD and modulate this regulation in an H_2O_2 responsive manner. These mechanisms are likely conserved within the ASK-family of kinases suggesting a common regulation of kinase signalling.

Investigating the Dynamic Consequences of Prostate Specific Antigen SNPs

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The current diagnostic test for prostate cancer measures serum levels of prostate specific antigen (PSA or KLK3) and has a high false positive rate. Genome wide association studies have identified two single nucleotide polymorphisms (SNPs) that are associated with a reduced risk of prostate cancer, and are therefore interesting from a diagnosis perspective. These coding mutations correspond to point mutations in PSA, a chymotrypsin-like serine protease, and lead to reduced activity and stability of the protein. In the absence of structures of the mutants, we made molecular models in order to understand the structural response to mutation. Predicted structural changes were insignificant therefore we hypothesized that mutations may exert their effects by altering conformational dynamics of the protein. We therefore performed molecular dynamics (MD) simulations of wildtype and mutant proteins, in triplicate. While the two main loops (the "kallikrein loop" and the "148 loop") of wild type PSA remain in their initial conformations over 500ns of simulation, they are disrupted in both mutants, triggering an increase in loop dynamics. We hypothesize that increased dynamics upon mutation triggers long-range conformational effects that ultimately disrupt the structure of the active site and therefore hinder the catalytic process (for example, substrate ingress/egress or transition state stabilisation). Analysis of the MD trajectories suggests that allosteric effects of mutation involve perturbations to electrostatic/H-bond networks. Our work may thus shed light on the molecular and physiologic consequences of these SNPs and assist the development of improved diagnostic tests for prostate cancer that utilise genetic information.

The activity of TRAF homo- and heterodimers is regulated by Zinc Finger 1.

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Ubiquitylation is a post-translational modification that relies on a tightly-regulated cascade of enzymes. Many immune signalling pathways rely on the synthesis of Lys63-linked ubiquitin chains to determine the strength, duration, and type of inflammatory response. One E3 ligase, TRAF6, plays critical roles in multiple immune signalling cascades. Dimeric TRAF6 builds Lys63-linked ubiquitin chains together with the heterodimeric E2 enzyme Ubc13-Uev1A, and these ubiquitin chains determine the strength of the response to ligand engagement of immune receptors.

We have solved two crystal structures of different length TRAF6 proteins in complex with a Ubc13~Ubiquitin conjugate. The structures show that TRAF6 RING dimers form a catalytic complex where the RING of one monomer interacts with Ubc13, while the Zinc Finger 1 (ZF1) domain and a linker-helix of the opposing monomer contact ubiquitin. Contacting residues were mutated and activity analysed to assess their importance for ubiquitin transfer. In addition, the dimer interface is conserved across many TRAFs and, by mixing of mutant TRAF proteins, we show that both TRAF homo- and TRAF heterodimers form.

The work presented here provides the first molecular explanation for the dependence of activity on TRAF RING dimers, and suggests that TRAF homo- and heterodimers are capable of synthesising ubiquitin chains. This study has important implications for understanding the complexity of ubiquitin signals generated downstream of immune signalling pathways.

Investigation the TRIM5α RING and B-box domains in self association in antiretroviral signalling

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The anti-retroviral restriction factor Trim5α prevents infection by diverse retroviruses, including HIV-1, disrupting early post-entry stages of the retroviral lifecycle[1]. As a member of the TRIM protein family Trim5α has a conserved N-terminal domain architecture consisting of an N-terminal RING domain with E3 ubiquitin ligase activity, and a B-box domain and antiparallel coiled-coil involved in self-assembly. To restrict a particular retrovirus, Trim5α must recognise the capsid of the incoming retrovirus. This recognition results in RING-mediated ubiquitylation, leading to the activation of downstream signalling events that induce a cellular antiviral state[2].

To better understand how capsid recognition translates into antiretroviral activity, we have studied the self-association and ubiquitylation activity of the RING and B-box domains. Oligomeric states were determined by size-exclusion chromatography with multi-angle light scattering and sedimentation velocity analytical ultracentrifugation. Ubiquitylation activity was tested with in vitro ubiquitylation assays involving E2 enzyme partners established in the literature. Previous work has established that a monomeric RING domain has no activity[3]. The RING-B-box construct exhibits higher order self-assembly and ubiquitylation activity. Mutations were made to disrupt self-assembly, which also resulted in a decrease in ubiquitylation activity. These results demonstrate that ubiquitylation activity is closely dependent upon Trim5α higher-order assembly, linking recognition of the retroviral capsid to ubiquitylation and the activation of restriction.

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