Abstracts submitted for the APG Awards 2018

Thaksaon Kittipassorn

Disruption of the glycolytic enzyme PKM2 decreases retinal MÃ¼llerglial cell number via glucose metabolism-independent mechanism

Pyruvate kinase M2 (PKM2) is an isoform of pyruvate kinase, acytoplasmic glycolytic enzyme. In some cancer cells, unlike itssplice-variant PKM1, PKM2 is proposed to promote proliferation anddrive cancer unique metabolism, termed aerobic glycolysis. PKM2 isalso the only isoform found in the nucleus and shown to act as acoactivator for the transcription factor hypoxia-inducible factor-1(HIF-1), leading to glycolysis upregulation. Furthermore, PKM2 is atarget gene of HIF-1, suggesting a feedback loop between the twoproteins. Surprisingly MÃ¼ller glial cells of the retina displaycancer-like aerobic glycolysis. Here we examine the hypothesis thatPKM2 plays a role in MÃ¼ller cell proliferation and metabolism. Firstwe verified the specificity of two PKM2 antibodies as PKM2 and PKM1only differ in a small mutually-exclusive exon, and found that onlyone binds to PKM2. Using the verified antibody, we show that PKM2 isexpressed in both the cytoplasm and nucleus of primary rat MÃ¼llercells and the MÃ¼ller cell line rMC-1. Additionally PKM2 expression inrMC-1 cells appears to be induced in hypoxia where HIF-1 is active.PKM2 knockdown decreases rMC-1 cell number, supporting a role for PKM2in MÃ¼ller cell proliferation. Interestingly the knockdown does notsignificantly affect glucose metabolism in rMC-1 cells. This indicatesthat the role of PKM2 in MÃ¼ller cell proliferation might not bemediated through glucose metabolism but may involve the HIF-1regulatory pathway. Given this role of PKM2, its disruption mightadversely affect MÃ¼ller cell survival and contribute to retinaldisease.

Kay Khine Myo Min

Desmoglein-2 as a target for tumour vasculature in melanoma

Tumour growth and cancer metastasis rely heavily on the ability ofcancer cells to gain access to nutrients and oxygen, and they achievethis via angiogenesis where endothelial cells (ECs) line up to formblood vessels. We have previously discovered that the adhesionmolecule desmoglein-2 (DSG2) promotes angiogenesis. Moreover, anincrease in DSG2 expression has been shown to correlate with poorprognosis in cancer, especially in melanoma. Thus our aim is todetermine whether DSG2 is a novel mediator of melanoma progressionthat can be therapeutically targeted. Our new data shows that in our syngeneic mouse model of melanoma, whena mouse melanoma cell line B16-F10 was injected into mice, tumourgrowth was attenuated in the desmoglein-2 loss-of-function mice(Dsg2lo/lo). An avenue that could be taken in order to counteracttumour growth is to reshape the tumour vasculature for improvedinfiltration of CD8+ cytotoxic T lymphocytes (CTLs). Increasing CTLsin the tumour can switch the vasculature to a high endothelial venule(HEV) phenotype, which has been reported to enable the infiltration ofCTLs, and correlates strongly with reduced tumour size.Histological analyses suggest that DSG2 may actively play a role inleukocyte infiltration in melanoma, as Dsg2lo/lo mice exhibit anincrease in HEVs, an increase in CD3+ T lymphocytes and a decrease inFoxP3+ T regulator cells involved in immune evasion. Upon inspectionof CD31+ vessels, Dsg2lo/lo mice also exhibited vessels with increasedEC width, suggesting that DSG2 is playing a role in regulating tumourvasculature. Further work is currently underway to determine DSG2â€™s involvementin regulating leukocyte infiltration and whether tumour vasculaturecan be â€˜switchedâ€™ to a high endothelial venule phenotype topromote infiltration of cancer killing T lymphocytes.

Debrah Renders

Nonsense Mediated mRNA Decay factors UPF3A and UPF3B have opposing roles on cell cycle in human embryonic stem cells.

Nonsense mediated mRNA decay (NMD) degrades transcripts with â€˜NMDinducing featuresâ€™. UPF3A and UPF3B are gene paralogs involved inNMD. UPF3B is a strong NMD activator, while UPF3As role is less clear,shown to have weak NMD activity and recently reported as an NMDinhibitor. Our aim was to study the role of UPF3B and UPF3A in hESCs.We used CRISPR-Cas9 genome editing technology to generate threeindependent knockout (KO) hESC lines each of UPF3A and UPF3B usingunique gRNAs. In UPF3B clones, UPF3A protein was elevated, identifyingexistence of a compensatory NMD mechanism previously described inother cells. In UPF3B clones, we observed increased mRNA levels ofbonafide canonical NMD targets and NMD factors consistent withimpaired NMD activity. Loss of either UPF3A or UPF3B had no overteffect on hESC morphology however the expression of SOX2 and OCT3/4were slightly reduced in the clones respectively. Cell cycle analysisrevealed that UPF3B clones had an enrichment of cells in the G1 phase,whilst in opposite, UPF3A clones had reduction in the G1 phase. Theexpression of NMD targeted cell cycle regulatory gene CDKN2A washighly upregulated only in UPF3B clones, and may in part explain thecell cycle defect. This data demonstrates that whilst UPF3A may bedispensable for canonical NMD in hESCs, it functions in anon-redundant, opposing manner to UPF3B to control hESC cell cycling.Our results demonstrate that the persistence of this only example ofNMD gene paralogs maybe due to their different roles in cells.

Aimee Horsfall

A Fluorescent Peptide Constraint to Define Secondary Structure

Protein-protein interactions are defined by interfacial secondarystructural motifs that impart a high degree of selectivity. Peptidescan be designed to bind at these sites, though they are invariablyunstructured, a shortcoming that can be addressed by introducing acovalent linker to define the required binding geometry. Imaging ofthese constrained peptides requires a fluorescent tag, however,classic constraints introduced by metathesis, lactamisation andâ€˜clickâ€™ chemistry lack this property and as such it must beintroduced separately. Here we repurpose a protein cross-linker,dibromobimane, as a peptide constraint to define secondary structurewith intrinsic fluorescence. A series of peptides with increasing length and terminal cysteineresidues were prepared by SPPS to investigate the effect of changingconstraint length on the peptide backbone geometry. These peptideswere then reacted with dibromobimane under biologically compatibleconditions to give a new class of fluorescent peptide with definedsecondary structure that can be directly imaged. All secondarystructures were characterised by NMR and CD to reveal an i-i+2homocysteine-containing peptide with beta-strand structure and asingle-turn helical geometry of an i-i+4 constrained peptide. Thefluorescent properties of the resultant constrained peptides wereinvestigated via plate photometry and detected at concentrations aslow as 10 nM, the peptides were also shown to be cell-permeable andimaged by confocal microscopy. Our new fluorescent peptide linker isintroduced in an efficient manner using natural amino-acid sequences,and allows the design of new protein-protein interaction inhibitorsthat do not require further functionalization for in vivo studies.

Ayla Orang

Harnessing miRNAs to enhance the anti-cancer properties of metformin in colorectal cancer

Colorectal cancer (CRC) is the third most prevalent cancer in theworld. Metformin linked to cancer prevention and selectively repressescancer progression. MicroRNAs are small non-coding RNAs involved inmost cellular processes. Although several metabolic effects ofmetformin treatment have been investigated, detailed analysis of theresultant changes in gene expression is still required. Also, theeffect of metformin treatment in combination with anti-cancer miRNAshas yet to be explored. RNA and small RNA next generation sequencing were performed for CRCcells treated with metformin. Following differential expression,functional enrichment and network analyses, CRC cells were transfectedwith miRNA mimics to explore the anti-cancer effect of differentiallyexpressed (DE) miRNAs. Also, high throughput functional screens ofmiRNA mimics library in combination with metformin were used andsecondary screen were performed to validate the hits.Protein-protein interactions and DE miRNAs and genes within specificbiological pathways that are resultant from metformin treatment wereidentified. Also, Metformin treatment resulted in downregulation ofsome pro-proliferative and upregulation of some anti-proliferativemiRNAs. Furthermore, miRNAs were validated to sensitize CRC cells tothe anti-cancer effect of metformin by inducing its anti-proliferativeeffects. Identification of DE miRNAs and their potential target genesas well as miRNAs that sensitise CRC cells to metformin provides a keystep towards identifying therapeutic innervation and confirms thefeasibility of combining metformin with miRNAs to enhance therapeuticefficacy and overcome drug resistance. Future work includesinvestigation of the mechanisms of action of newly discovered miRNAsin the context of metformin.

Andrew Thompson

Alternate binding modes facilitate nucleoside promiscuity in MtDTBS

The rise in resistant tuberculosis (TB) infections must becombated with the development of new antibiotic therapies. Theeponymous pathogen, Mycobacterium tuberculosis (Mt), relies onbiosynthesis to generate the essential nutrient biotin, making this apromising pathway for anti-TB antibiotics. Dethiobiotin synthetase(DTBS) catalyses the penultimate step of biotin synthesis â€“ theenergy-dependant conversion of diaminopelargonic acid (DAPA) todethiobiotin. Uniquely, MtDTBS can promiscuously utilise allnucleoside triphosphates (NTPs) for catalysis, with a preference forcytidine triphosphate (CTP).In this work, improved surface plasmon resonance (SPR) protocols aidedthe quantitative determination two modes of NTP binding. TheCTP-MtDTBS complex was high affinity and exhibited slow dissociation.In contrast, other â€œpromiscuousâ€ NTPs formed transient, loweraffinity complexes with fast kinetics.Crystallographic studies helped define the hydrogen bonding networkresponsible for the high affinity binding mode, which was observed inseveral ligand complexed crystal structures including:cytidine-MtDTBS, cytidine diphosphate-MtDTBS, andCTP-DAPA-carbamate-MtDTBS. However, structural investigation of otherNTP binding modes was prohibited by competitive binding withcrystallographic precipitant molecules. To overcome this, aprecipitant-ligand exchange technique was developed and 4 NTP-MtDTBScrystal structures were solved. These structures revealed thatpromiscuous NTPs bound exclusively to MtDTBS via the triphosphategroup, a distinct mechanism from that observed for CTP. These data provide the structural basis for the promiscuousutilisation of NTPs and an unconventional mechanism of enzymecatalysis. These findings enhance our overall understanding of theMtDTBS active site and will guide the rational design of inhibitors totarget this essential TB enzyme.

Byron Shue

Identification of RACK1 as a critical pan-flavivirus host factor for virus replication using CRISPR/Cas9 screening technology

The Flaviviruses, such as Zika virus (ZIKV), Dengue virus (DENV) andWest Nile Virus are significant human pathogens that cause substantialburden on society, particularly in the developing world. Cellularfactors play important roles in all facets of the flavivirus lifecycle and deciphering viral-host protein interactions is essential forunderstanding the flavivirius lifecycle and development of effectiveantiviral strategies.

To identify novel ZIKV essential host factors, we employed aCRISPR/Cas9 genome-wide KO screen approach utilising single guide RNAs(sgRNAs) targeting every gene in the human genome. sgRNAs from cellswhich survive the ZIKV induced cytopathic effect were PCR amplifiedand sequenced using Illumina NextSeq. Bioinformatics analysisidentified a highly enriched cohort of sgRNA sequences representingknockout of genes critical for ZIKV replication. One host factor,RACK1 was significantly enriched across multiple screens. RACK1 playsmultiple roles in homeostatic cellular processes and has beenpreviously identified as essential for replication of severalunrelated viruses (HCV, Pox virus).

siRNA knockdown of RACK1 followed by infection with ZIKV or DENVrevealed that RACK1 has a critical role in replication for not onlyZIKV but other members of the flavivirus family. Furthermore, RACK1was shown via co-immunoprecipitation and proximity ligation assay tointeract with numerous ZIKV non-structural proteins which areimportant for establishing the viral replication complex. Takentogether, it is likely that RACK1 may act as a platform for therecruitment of non-structural proteins to the replication complex inthe ER during early stages of flavivirus replication.

Alana Donnelly

Catalytically inactive dCas9 as a transcriptional roadblock to modulate gene expression

A small number of DNA-binding proteins are known to hinder themovement of an elongating RNA polymerase along DNA by acting as aphysical roadblock. These roadblocking proteins make promising genomeengineering tools, with applications such as loss-of-function geneticscreening and construction of synthetic gene networks. Studies haveshown that a catalytically inactive Cas9 enzyme (dCas9) can reduce thelevel of gene expression by acting as a transcriptional roadblock. Thesimple two-component Cas9 system and the ability to target virtuallyany gene, makes dCas9 a promising tool for programmable roadblocking.The effects of a number of cellular conditions, such as dCas9concentration, promoter strength of the target gene and orientationand affinity of dCas9 binding, on dCas9 roadblocking are not yet fullyunderstood. Through in vivo testing using simple modular systemswithin in E. coli cells, we showed how increasing the promoterstrength of the target gene reduces the repressive effect of dCas9,that increasing concentration of dCas9 in the cell enables greaterroadblocking and that the correct orientation of dCas9 binding on theDNA is vital for effective transcription repression by dCas9. Withthis data, we aim to develop a mathematical model which will allow usto extract biochemical parameters describing roadblock kinetics toassist improved manipulation of gene expression through optimisingroadblocking conditions.

Charlotte EJ Downes

Identification and computational modelling of ruxolitinib resistant mutations in JAK2-rearranged B-cell acute lymphoblastic leukaemia

JAK2 rearrangements (JAK2r) occur in approximately 5% ofpaediatric B-cell acute lymphoblastic leukemia (B-ALL) patients andare associated with poor prognosis. A clinical trial is currentlyassessing the Jak1/2 inhibitor, ruxolitinib (rux) in high-risk B-ALLcases harbouring JAK2 pathway alterations. Elucidating mechanisms ofrux resistance in JAK2r B-ALL will enable the development oftherapeutic strategies to overcome or avert resistance. JAK2r B-ALLwas modelled in the pro-B cell line, Ba/F3, by expressing thehigh-risk B-ALL fusion, ATF7IP-JAK2. Rux resistance was generatedfollowing dose escalation to a clinically relevant dose of 1 ÂµM inthree independent experiments. Sanger sequencing of RT-PCR amplifiedJAK2 fusion specific transcript revealed each resistant line hadacquired a different mutation within the JAK2 kinase domain,suggesting that mutation-based resistance was stochastic. In additionto the identification of two known rux resistant mutations, JAK2p.Y931C and p.L983F, a novel p.G993A mutation was also detected.Computational modelling of acquired JAK2 mutations and their influenceon rux binding was performed using ICM-Pro (Molsoft L.C.C.). Themutations localised to the ATP/rux binding site of the kinase domain.JAK2 p.L983F sterically hinders rux binding, while JAK2 p.Y931C mayreduce rux binding affinity by disruption of a critical hydrogen-bondwithin the ATP-binding site. Interestingly, the novel JAK2 p.G993Amutation is predicted to alter DFG-loop dynamics by stabilising theJAK2 activation loop, potentially altering kinase activity.Understanding mechanisms of rux resistance, as modelled here, has thepotential to inform future drug design in this high-risk patientcohort.

Alexander Lewis

Dual sphingosine kinase and Bcl-2 inhibition exhibits synergistic cell death in acute myeloid leukemia

Pro-survival Bcl-2 family proteins such as Mcl-1 and Bcl-2 havegarnered significant interest as therapeutic targets due to theirup-regulation in many cancers, including acute myeloid leukaemia(AML), leading to enhanced cancer cell survival. Small moleculeinhibitors such as the selective Bcl-2 inhibitor, Venetoclax, are veryeffective in some cancers that are highly dependent on Bcl-2, but havedemonstrated poor single agent efficacy in AML due to these cellsbeing highly dependent on Mcl-1. Sphingosine kinase 1 (SK1) is asignalling enzyme with established roles in oncogenesis and hasrecently emerged as a potential therapeutic target in leukaemia. Werecently demonstrated that the selective SK1 inhibitor, MP-A08exhibits anti-leukemic activity in vitro and in vivo using patientderived AML xenograft models. MP-A08-mediated cytotoxicity in AMLcells correlated with a reduction in Mcl-1 levels, as well asupregulation of BH3 only proteins. Here, we found that combinationaltherapies with MP-A08 and Venetoclax induced synergistic cell death inAML cell lines and patient samples. Mechanistically, MP-A08 inducestranscriptional upregulation of BH3 only protein, Noxa and formationof Noxa/Mcl-1 complexes. MP-A08 appears to exert its cytotoxicity inAML cells through loss of Mcl-1 as a consequence of Noxa binding.Combining MP-A08 and Venetoclax significantly reduced leukemic burdenin a patient derived AML xenograft model. This data providespre-clinical evidence to investigate dual MP-A08 and Venetoclax as apotential therapeutic strategy in AML.

Stephanie Nguyen

Structural insights into the essential Aspergillus fumigatus enzyme, nucleoside diphosphate kinase.

Invasive aspergillosis is a serious infection commonly occurring inimmunocompromised patients and is often caused by the fungus,Aspergillus fumigatus. The increasing resistance to currentlyavailable antifungal therapies and the high mortality rate of invasiveaspergillosis highlights the need to characterise novel antifungaltargets in A. fumigatus. Previously demonstrated to be essential forviability in A. fumigatus, nucleoside diphosphate kinase (NDK) isvital in maintaining the nucleotide triphosphate pool for DNAsynthesis and therefore poses as an attractive antifungal drug target.Two atomic structures of this novel antifungal target were solvedusing X-ray crystallography, revealing a hexameric arrangement of theenzyme as a stack of two trimers. Comparison between the A. fumigatusNDK structure in apo state (2.0 Ã…) and bound to both ADP andmagnesium (2.2 Ã…) reveals the movement of a loop containing residuePhe59 that forms a pi-pi stacking interaction with the adenine of ADPto position the product in the active site. This study provides astructural foundation for rational drug design of inhibitors of NDK.Future work is planned to involve co-crystallisation of NDK with othernucleotide triphosphates and diphosphates, ATP analogues and knowninhibitors to obtain a more comprehensive understanding of the bindingmechanism.

Jia Truong

Crystal structures of an unusual transcriptional activator from bacteriophage 186

The temperate coliphage 186, after infecting its host bacteriumEscherichia coli, can follow either the lytic or the lysogenicdevelopmental pathways. Crucial to this developmental decision is thelysogeny promoting factor CII. This potent transcriptional activatoractivates the early lysogenic promoter pE at least 400 fold, to buildup sufficient immunity repressor levels for a portion of infections tocommit to lysogeny. Its potency and its unusual property of binding tohalf sites separated by 20 base pairs, center-to-center, suggests itmay activate the pE promoter by a novel mechanism. Three crystalstructures of the CII protein were solved to 2-3Ã…. The structuresreveal that a tetrameric arrangement of CII is necessary for DNAbinding, which was subsequently validated by mutational analysis andnative mass-spectrometry. CII is degraded in vivo into a specifictranscriptionally inactive product. The crystal structures explain thealtered self-association of the degradation product and its loss ofactivity. The structures combined with mutagenesis data provide abasis for modelling the CII-RNA polymerase complex at the promoter toaid in understanding the promoter activation mechanism.

Pawanrat Tangseefa

Metabolic and reproductive abnormalities in mice with impaired skeletal-mTORC1 function mirror a dietary restriction phenotype

Dietary restriction (DR) improves whole-body metabolism, and reducesreproductive function. While the mechanisms leading to these profoundphysiological changes remain to be elucidated, suppression of mTORC1is thought to play a critical role. The skeleton has recently emergedas a critical endocrine tissue that regulates glucose and energymetabolism and male reproductive function, via release of thebone-specific hormone osteocalcin (OCN), suggesting that suppressionof mTORC1 in the skeleton could play a crucial role in thephysiological responses to DR.To investigate the role of skeletal-mTORC1 in modulating glucosemetabolism and male fertility, we generated mice in which raptor, anessential component of mTORC1, is specifically deleted in osteoblasts(RaptorOB-/-). RaptorOB-/- mice are significantly smaller thancontrols, have increased bone marrow adipose tissue (MAT) and reducedserum OCN levels. Compared to controls, serum adiponectin levels aresignificantly elevated in RaptorOB-/- animals, while leptin levels arereduced. Importantly, despite being hypoinsulinemic, RaptorOB-/- micehave significantly lower fasting glucose levels, suggestive of insulinhypersensitivity. Consistent with this, insulin and glucose tolerancetests have revealed that RaptorOB-/- mice have improved glucosetolerance, enhanced insulin sensitivity and elevated insulinsecretion. Furthermore, the reproductive function of male RaptorOB-/mice is significantly impaired, as evidenced by reduced circulatingtestosterone levels and sperm counts. Collectively, our resultsdemonstrate that physiological changes associated with DR (e.g.elevated MAT and circulating adiponectin levels, reduced leptinlevels, improved glucose metabolism and low testosterone levels) aremirrored in RaptorOB-/- mice, which suggests that skeletal mTORC1signalling is critical in mediating cellular responses to DR.

Ellen Potoczky

PR/Set domain 5: A Critical Transcriptional Regulator of Craniofacial Development

Development of the craniofacial skeleton is dependent on complexcellular processes involving cellular migration, differentiation andmorphogenesis. Disruption of these processes can result incraniofacial defects which have a significant impact on quality oflife. To identify the genes andmolecular pathways involved in craniofacial development, anN-ethyl-N-nitrosourea mutagenesis forward genetic screen was performedin zebrafish. From this screen, a novel zebrafish line withcraniofacial defects was discovered, which contained a mutation in thePRDM5 gene. Interestingly, PRDM5 mutations have been identified inhumans with Brittle Cornea syndrome,where patients exhibit corneal thinning and severe corneal ruptureswith associated hearing loss. Alcian blue staining of this uniquezebrafish line revealed chondrocyte stacking defects and a morphogenicchange in Meckelâ€™s and palatoquadrate cartilage of the lower jaw. Aschondrocytes are a Neural Crest derivative, these cartilage defectssuggest a role for PRDM5 in Neural Crest cell development. Comparativeexpression analysis revealed no significant changes in Neural Crestinduction and migration, however a reduction in Collagen Type 2 alpha1 (Col2a1) was identified in homozygous mutants, suggesting that PRDM5normally acts as a positive regulator of Col2a1 expression incartilage precursors. Given the homology between the proximal portionof Meckelâ€™s cartilage and the palatoquadrate cartilage of zebrafishwith the incus and malleus of the middle ear in mammals, this dataprovides new insight into the mechanisms by which PRDM5 mutationscontribute to the etiology of Brittle Corneasyndrome.

Rebecca Frkic

Optimisation of PPARÎ³ Partial Agonists for Improved T2DM Therapies

Peroxisome Proliferator-Activated Receptor Î³ (PPARÎ³) is aligand-activated nuclear receptor which plays a key role in fatty acidand glucose homeostasis. PPARÎ³ is the molecular target for type 2diabetes mellitus (T2DM) therapeutics known as the TZDs(thiazolidinediones), drugs that offer robust clinical benefit interms of normalising fasting glucose. However, the TZDs, which arefull agonists of the receptor, have been confounded with significantside effects. In recent years, it has been shown that partial agonistsof PPARÎ³ have displayed similar insulin sensitising efficacy as thefull agonist TZDs, but lack many of the undesirable side effects ofthe full agonists. One such partial agonist, INT131, has shown potentinsulin-sensitising actions with reduced side effects as compared tothe TZDs. To probe the structure-activity relationship (SAR) of theINT131 scaffold, 14 analogues of INT131 were synthesised. SAR studiesof the analogues revealed compounds with higher transcriptionalpotency for PPARÎ³ as well as identification of moieties of the INT131scaffold key to high transcriptional potency. The sulphonamide linkeris absolutely critical to activity, substitutions at position 4 of thebenzene ring A were associated with higher transcriptional activity,substitutions at position 2 of benzene ring A aided in tighter packingand activity, and the ring type and size of ring A was correlated tothe degree of activity.

Ruby Dawson

A conditional mouse model of GATOR1-related focal epilepsy supports a second-hit mechanism of disease

Heterozygous mutations in DEPDC5, NPRL2 and NPRL3, which encode theGATOR1 complex, have been found in families with focal epilepsy.However the pathological mechanism of how these mutations cause thedisease remains elusive. GATOR1 functions to inhibit mTOR signallingand hyperactivity of this pathway has independently been linked withepilepsy. Therefore, mTOR pathway deregulation is hypothesised to bethe major factor in the pathology of GATOR1-related epilepsy.

We developed a functional assay using CRISPR null cell lines, wheremutations are screened based on their ability to rescue the nullphenotype of hyperactive mTOR. Several mutations have been confirmedto have lost this function, supporting a link between mTORderegulation and the disease. Interestingly, many of the missensemutations screened retained their function, highlighting that somepredicted disease-causing mutations may not be pathogenic.

We aimed to further investigate the disease pathology using a mousemodel. Following our initial finding that Depdc5 heterozygous mice donot display any human disease phenotypes, we are now investigating a'second-hit' mechanism of disease. This is where seizures are proposedto result from a second, somatic mutation in the brains of germlineheterozygotes, resulting in null cell clones. This mechanism issupported by the variable foci, incomplete penetrance of the diseaseand the finding of a somatic mutation in DEPDC5 in one patient. Wetherefore established a conditional mouse for Depdc5 to model thismechanism.

Using CRISPR, we generated a floxed allele which, following theunilateral electroporation of Cre into developing brains, recombinesto result in discrete regions of null tissue. We observed increasedmTOR signalling and increased soma size in DEPDC5 null neurons. We areperforming seizure threshold testing to determine if these molecularchanges translate to disease phenotypes. Data from mutant cell lineand mouse investigations support the involvement of mTOR deregulationin GATOR1-related epilepsy and a second-hit disease mechanism.

Cameron D. Haydinger

Molecular drivers of aerobic glycolysis in the mammalian retina

Most glucose metabolised by the mammalian retina is converted tolactate after glycolysis despite the availability of adequate oxygenfor complete breakdown by oxidative phosphorylation. This type ofmetabolism, known as aerobic glycolysis or the Warburg effect, iscommon in highly proliferating cells including cancer cells, but rarein non-proliferating tissues such as the retina. Several pathways areknown to drive aerobic glycolysis in cancers, but those that drive itin the retina are unknown. To elucidate drivers in the retina, wetreated cultured immortalised MÃ¼ller cells, a type of retinal glialcell that displays aerobic glycolysis, with inhibitors of proteinsknown to drive the process in cancers. Extracellular acidificationrate (ECAR) and oxygen consumption rate (OCR) were measured using anextracellular flux analyser, informing lactate production andoxidative phosphorylation rates, respectively. When treated with aninhibitor of a small GTPase, Rac1, MÃ¼ller cells had a dramaticallyreduced ECAR without a simultaneous decrease in OCR, indicating thatRac1 drives aerobic glycolysis in these cells. A partial decrease inlactate production was also observed when MÃ¼ller cells were treatedwith PI3K inhibitors in the presence of serum, and this effect wasindependent of PI3Kâ€™s canonical downstream signal mediator Akt. Weare currently exploring the role of Rac and related signallingpathways in vivo. Dysregulated glucose metabolism is a possible causeof blinding diseases such as diabetic retinopathy. By understandingthe protein pathways that drive aerobic glycolysis in the retina, wemay be able to unravel specific causes of disease and develop newtargeted treatments.

Yu Chinn Joshua Chey

Sn'HIFâ€™fing out HIF in Multiple Myeloma

Multiple myeloma (MM) is a plasma cell (PC) dyscrasia characterisedby the abnormal proliferation and dissemination of PCs throughout theskeleton. As the bone marrow microenvironment is well-established tobe hypoxic, hypoxia is thought to promote tumour growth, angiogenesis,metastasis and bone osteolysis. Major cellular transcriptional changesin response to hypoxia are mediated through the heterodimericHypoxia-Inducible Factors (HIFs). The two main isoforms of theoxygen-regulated HIF alpha subunit, HIF-1Î± and HIF-2Î±, mediate bothoverlapping and disparate responses in a tumour-specific context.While the HIFs have been implicated in the progression of MM, theirdistinct roles have not been well elucidated. To explore each HIFÎ±isoformâ€™s role in the context of MM, we used CRIPSR/Cas9 technologyto develop both doxycycline (dox)-inducible and constitutivelyknocked-out monoclonal MM cells for both HIF1Î± and HIF2Î± in 5TGM1murine MM cells. Using a modified lentivirus gRNA plasmid with anmPlum selection marker, we have successfully generated inducible HIFÎ±knock-out cells at a high transduction efficiency. Induction ofCRISPR/Cas9 activity occurs specifically on dox-treatment andconstitutively knocked-out monoclonal MM lines were sequence verifiedby sanger sequencing. Ultimately, we aim to use pooled monoclonal MMcells to study the effects of each HIFÎ± knockout on MM diseaseprogression and dissemination in the syngeneic KaLwRij mouse model.Extending our limited understanding of HIFâ€™s roles in MM will helpbuild the rationale for targeting the hypoxic BM niche and for therepurposing of HIF inhibitors in MM therapy.

Marina Zupan

Elucidating the Zn(II)-binding mechanism of the pneumococcal protein AdcAII.

Streptococcus pneumoniae is a globally significant human pathogenresponsible for 1 â€“ 2 million deaths annually. To colonise andpersist within the host, the bacterium must acquire the transitionmetal ion zinc [Zn(II)], present at low concentrations in the hostenvironment. In S. pneumoniae, Zn(II) import is facilitated by theATP-binding cassette transporter, AdcCB, and two Zn(II)-specificsolute binding proteins, AdcA and AdcAII. While AdcA and AdcAII bothdeliver Zn(II) to the AdcCB transporter, AdcAII has a more criticalrole for survival under Zn(II) starvation. Although its importance toZn(II) acquisition is well-characterised, the molecular details of howthe protein selectively acquires Zn(II) remain poorly understood.Structural information is currently available for Zn(II)-bound AdcAII,however, our understanding of the Zn(II)-binding mechanism of theprotein is limited by the lack of an open, metal-free crystalstructure. In this study, we overcame this issue by mutating each ofthe Zn(II)-coordinating residues of AdcAII and performing structuraland biochemical analyses. Structural analyses of the Zn(II)-boundAdcAII variant isoforms revealed how specific regions within theprotein undergo conformational changes via their direct coupling toeach of the metal-binding residues. Complementing this work,metal-binding studies revealed that mutagenesis of the coordinatingresidues altered both the metal ion selectivity of the protein and itsaffinity for Zn(II). Collectively, these results provide new insightinto the mechanism of Zn(II)-binding by AdcAII and the biophysicalbasis by which the protein confers selectivity for this essentialmetal ion.

Daniel Saviane

Elucidating the importance of alpha-macroglobulin dimers in innate immunity

Alpha-macroglobulins (Î±Ms) are a highly conserved family ofsecreted proteins. The predominant Î±M family member in humans isalpha-2-macroglobulin (Î±2M), which is best known as a proteaseinhibitor, but can also stabilise misfolded proteins, facilitate theclearance of bacteria and influence many signalling pathways. NativeÎ±2M is a tetramer, but is induced to dissociate into dimers byhypochlorite, an oxidant generated during inflammation. Additionally,during pregnancy and inflammation, pregnancy zone protein (PZP), whichshares ~70% amino acid sequence identity with Î±2M, is markedlyupregulated. Compared to what is known about the native Î±2M tetramer,far less is known about the biological importance of dimeric Î±Ms. Theoverarching goal of this project is to characterise the functions ofÎ±2M dimers, including those generated using drug-like smallmolecules. Preliminary studies show that (i) Î±2M dimers are generatedby FDA-approved n-acetyl cysteine and (ii) compared to the native Î±2Mtetramer, hypochlorite-modified Î±2M dimers preferentially bind to thesurface of group A Streptococcus bacteria. A better understanding ofthe functions of Î±2M dimers will help us to determine whether or notincreasing their levels will have therapeutic benefits in infectiousdiseases and beyond.

Saira Ali

Long non-coding RNA-protein interactions and butyrate sensitization of colorectal cancer cells

Colorectal cancer (CRC) is the second most common cause of Australiancancer related deaths. The development of CRC is associated withepigenetic alterations including altered histone acetylation patternsand dysregulated long non-coding RNA (lncRNA) expression. Butyrate, ashort-chain fatty acid, produced from the fermentation of dietaryfibre in our gut, has been shown to alter CRC cell behaviour throughepigenetic mechanisms. Butyrate can alter CRC gene expression,including lncRNA expression, via histone deacetylase inhibitionactivity, resulting in decreased proliferation and increasedapoptosis. lncRNAs regulate gene expression through various mechanismsincluding epigenetic modifications, lncRNA-miRNA, lncRNA-mRNA,lncRNA-protein interactions and their ability to produce regulatoryncRNAs, such as miRNAs. lncRNAs have been shown to regulate cellgrowth and apoptotic pathways in CRC. The effect of exposing CRC cellsto the anti-tumorigenic molecule, butyrate, in combination with lncRNAknockdown has yet to be investigated. High throughput functionalscreens were used to systematically identify oncogenic lncRNAs, whichwhen knocked down resulted in the sensitization of CRC cells tobutyrate (enhanced anti-proliferative and pro-apoptotic effects).Knockdown of some lncRNAs resulted in enhanced apoptosis in thepresence of butyrate. Pathway and network analyses assisted inidentification of predicted key lncRNA-protein interactions involvedin apoptosis. Further investigation of lncRNA knockdown and theirprotein interactors in the context of butyrate is required.Identification of oncogenic lncRNAs, and protein interactors, with theability to sensitise CRC cells to butyrate when suppressed, may revealthe potential chemo-preventive or therapeutic value of thesebiological molecules.

Joseph Rossi

Utilising a Tri-Partite Split GFP Assay to Characterise Novel Protein-Protein Interactions

The basic Helix-Loop-Helix PER-ARNT-SIM (bHLH-PAS) transcriptionfactor Single Minded 1 (SIM1) is integral to hypothalamic development,and through maintained expression within these neurons regulatessatiety. Orthopedia (OTP) is a homeodomain containing transcriptionfactor which is expressed in similar regions of the hypothalamus toSIM1 and has an analogous role in hypothalamic development andsatiety. Importantly, mutations in both factors have been linked tohyperphagic obesity in human patients. We have recently demonstratedby immunohistochemistry that SIM1 and OTP are indeed expressed in thesame neurons of the hypothalamus. Through co-immunoprecipitation(Co-IP) experiments, we then identified the existence of a SIM1/OTPcomplex. Additional Co-IP experiments have started to elucidate thedomains of interaction between SIM1 and OTP. To further characterisethe novel interaction, we have employed the Tri-partite Split GFPsystem developed by Cabantous et. al. (DOI: 10.1038/srep02854). Inthis system the two proteins of interest are each uniquely tagged witha small GFP fragment. The remainder of the GFP protein not included inthe tags forms a third fragment. If the two proteins interact, thesmall GFP tags will form a complex capable of interacting with thelarge GFP fragment, forming a complete GFP molecule capable ofproducing green fluorescence. Preliminary data for the Tri-partiteSplit GFP assay has confirmed the interaction between SIM1 and OTP, aswell as providing information on the likely cellular localisation ofthe interaction. We have started to characterise a novel interactionbetween SIM1 and OTP that may have implications in appetite controland obesity.

Melissa Bennett

Inhibition of glucosylceramide synthase causes multiple myeloma cell death alone and in synergy with bortezomib via enhanced endoplasmicreticulum stress

Ceramide is an apoptotic sphingolipid which is often elevated in cellsby chemotherapy and radiotherapy, and contributes to the cell deathcaused by these agents. Some cancers are able to avoid thepro-apoptotic signalling produced by ceramide by upregulating enzymeswhich are able to convert ceramide to less apoptotic sphingolipids.Glucosylceramide synthase (GCS), which converts ceramide toglucosylceramide, is one such enzyme. One cancer in which GCS seems tobe important is multiple myeloma, a currently incurable blood cancerwhich arises from plasma cells. GCS is significantly upregulated inpatient myeloma cells compared to normal plasma cells. Inhibition ofGCS, both genetically through shRNA, and pharmacologically through theGCS inhibitor PDMP, causes cell death of myeloma cell lines, asmeasured by flow cytometry with Annexin-V/PI staining. This cell deathwas accompanied by an increase in markers of endoplasmic reticulum(ER) stress and caspase-3 cleavage, suggesting the mechanism of celldeath is apoptosis induced by enhanced ER stress. Furthermore,combining PDMP with the proteasome inhibitor bortezomib, which iscurrent first line therapy for MM, is able to cause synergistic celldeath of MM cell lines, even in cell lines that are bortezomibresistant, with was associated with synergistic induction of ERstress. Given that bortezomib resistance is a substantial hurdle inthe treatment of MM, the ability of PDMP to improve bortezomibresponse in these cells is highly significant. Therefore, it seemsthat GCS inhibition may be a viable target in multiplemyeloma.

Erin Brazel

Overcoming antimicrobial resistance â€“ exploiting zinc intoxication to restore antibiotic efficacy

The prevalence of antibiotic resistant pathogens continues to rise andthreatens to disrupt healthcare on a global scale. To combatantibiotic resistance, novel strategies for treating bacterialinfections are urgently required. The metal ion zinc has a criticalrole in innate immune defense and its deficiency is associated with amarked increase in susceptibility to bacterial infections.Streptococcus pneumoniae is a major cause of local and invasivediseases and is associated with significant human mortality. Despitethe importance of zinc at the host-pathogen interface, the impact ofzinc stress on S. pneumoniae remains poorly understood. Here, weinvestigated how zinc stress affected the virulent S. pneumoniae D39strain using a combination of phenotypic growth, cellular metalaccumulation and macrophage survival analyses. These studies revealedthat S. pneumoniae encoded a cation diffusion facilitator familytransporter (CzcD) that was capable of zinc efflux and contributed topneumococcal survival within phagocytic cells. We further examined theimpact of zinc stress by abolishing czcD functionality. This revealedthat zinc intoxication rendered S. pneumoniae more sensitive tospecific classes of antibiotics. Building on these findings, weexamined synergism between zinc and antibiotics using ionophores toincrease the potency of zinc stress. Ionophore-mediated zinc treatmentrestored antibiotic susceptibility to the multidrug resistant S.pneumoniae 23F strain. Collectively, this study provides detailedinsight into zinc resistance in S. pneumoniae and highlights thetherapeutic potential of zinc and ionophores as adjuvants toantibiotics as a novel treatment strategy.

MD Raihan sarkar

The investigation of new monoxygenase enzymes as biocatalyst for selective C-H bond oxidation reactions

The cytochrome P450 enzyme CYP101B1 from the bacterium Novosphingobiumaromaticivorans DSM12444 binds and oxidises norisoprenoids and otherstructurally diverse classes of substrate. CYP101B1 catalyses theoxidation of norisoprenoids such as Î²-ionone with high activity andcoupling efficiency. Substrate engineering using acetate, isobutyrate,amide directing groups with the aim of mimicking the butenone sidechain of the norisoprenoids was undertaken with cyclic alcohol andterpenes. This approach significantly increased the affinity, activityand coupling efficiency of CYP101B1 for the esters/amides compared tothe parent alcohol or amine. The majority of the turnovers were regio-and stereo-selective for C-H bond hydroxylation. CYP101B1 is also ableto bind and oxidise aromatic substrates but at a lower activity andefficiency than norisoprenoids and monoterpenoid esters describedabove. Based on protein sequence alignments we hypothesised the sidechain of histidine 85 would interact with the carbonyl groups of thefavoured norisoprenoid substrates of CYP101B1. Site directedmutagenesis of residue histidine 85 of CYP101B1 to phenylalanine (F)was used with the aim of improving the activity of the enzyme for morehydrophobic substrates. The H85F variant of CYP101B1 showed enhancedaffinity and activity towards alkylbenzenes, styrenes andmethylnaphthalenes. Finally the affinity and activity of CYP101B1 fora series of 4-methylcubane derivative was also determined. Thesesubstrates would be held in the substrate binding pocket of CYP101B1in such a fashion that the methyl (or ethyl) C-H bonds would be closeto the heme iron enabling efficient and selective abstraction to occurat this position. The oxidation of cubane at this position wouldenable an assessment of the nature of any carbon based radicalintermediate formed during this process and allow us to probe themechanism of this important family of enzymes.

Shaghayegh (Sherry) Dezvarei

Conversion of a versatile monooxygenase into a peroxygenase by asingle mutation

The cytochrome P450 family of monooxygenase enzymes oxidise unreactiveC-H bonds, by activating molecular dioxygen to form a reactiveiron-oxo intermediate. CYP102A1 from Bacillus megaterium (P450Bm3) isone of the most utilized P450 enzymes in biocatalytic hydroxylationstudies because of its high solubility and activity. Moreover, thefusion of reductase and heme domain makes it a self-sufficient enzymewhich just needs NADPH cofactor as a source of electrons. A mutantvariant of CYP102A1, R19 (R47L/Y51F/H171L/Q307H/N319Y) was generatedwhich shifts the substrate range away from fatty acids, which areoxidised by the wild-type enzyme, to range of other substrates. Theactivity of this variant was tested with styrene, ethylbenzene andthioanisole and an enhancement of 100-, 50- and 3-fold, respectively,compared to the WT enzyme was found. The activity was further improvedby the co-addition of fluorinated fatty acid like decoy molecules butthe selectivity of the enzyme was maintained.One of the drawbacks of using these monooxygenase enzymes asbiocatalysts is the high cost of NADPH, which limits theirapplication. Inspired by CYP152A1, a natural H2O2-dependentperoxygenase enzyme, a single mutation of threonine 268, which isinvolved in dioxygen activation, to glutamic acid was made to haemdomain of CYP102A1. This mutant was found to convert the enzyme into ahydrogen peroxide (H2O2) dependent variant. This variant displayedoxidation of the aforementioned substrates with H2O2. The addition ofdecoy molecules did not improve activity, with the T268E peroxygenasebut was found to alter the stereo-selectivity of ethylbenzeneoxidation.

Tom Coleman

Structural investigation of the role of the substrate and the acid-alcohol pair of residues in dioxygen activation by cytochromeP450 enzymes; with insight into the active oxidant(s) employed byCYP199A4.

The ubiquitous cytochrome P450 superfamily of enzymes catalyse theinsertion of an oxygen atom into usually unreactive C-H bonds. Theseenzymes are critical for mammalian xenobiotic metabolism, as well asnatural product biosyntheses. These enzymes use molecular dioxygenwhich is activated to form the reactive intermediate, Compound I (CpdI; PorFeIV=O) [1]. The details of oxygen activation are basedpredominantly on structural work from a single enzyme, P450cam(CYP101A1). Mutation of the highly conserved pair of acid-alcoholresidues, Asp251 and Thr252, suggested that the threonine plays a rolein stabilising the iron-oxy intermediate, while the aspartate isthought to facilitate delivery of protons to the active site [2].

Here we use the bacterial CYP199A4 P450 enzyme, from the metabolicallydiverse Rhodopseudomonas palustris HaA2, to shed further insight intothe mechanism of oxygen activation and substrate oxidation by P450enzymes. CYP199A4 catalyses the regioselective oxidation ofpara-substituted benzoic acid substrates. It can accept a wide varietyof functional groups and perform a wide variety of oxidations, such ashydroxylation, oxygen/nitrogen dealkylation, desaturation, and sulfuroxidation.

Crystal structures of CYP199A4 were solved, in which the equivalentacid-alcohol pair of residues were mutated. These indicated thatCYP199A4 is less sensitive to mutation induced structural changes thanP450cam. In addition, turnover studies were performed which revealedunexpected high levels of product formation for these mutants. Theseresults prompt further discussion on whether the mechanistic detailsof P450cam can be extrapolated to other systems.

References

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