International Dictyostelium meeting 2022

7-11th August

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Sunday 7th August

6:00-7:40pm **Chemotaxis 1 (Chair: Allyson Sgro)**

1. Jonathan Kuhn: Cortical actomyosin suppresses directed migration signaling networks
2. Hugh Ford: Establishing the coding properties of spiral waves
3. Xuehua Xu: Ras inhibitors gate chemoattractant concentration range for chemotaxis in eukaryotic cells

Plenary: Will Wood, University of Edinburgh: Motility in wound-healing and morphogenesis

7:45pm Reception (buffet dinner and drinks)

Rob Kay, reflections on Jeff Williams

Monday 8th August

9-10:40. **Development (Chair: Pauline Schaap)**
4. Tetsuya Muramoto: CRISPR/Cas9-based genome-wide screening of *Dictyostelium*
5. Satoshi Kuwana: The most important time in Dicty's life
6. Elizabeth Westbrook: Excitable signalling drives a discontinuous developmental transition
7. Chris Brimson: Evidence that the cAMP signalling system provides a coupled oscillator and allows developmental time to be encoded
8. Chris Thompson: Information averaging facilitates symmetry breaking, lineage commitment and developmental robustness

Coffee: 10:40-11:10

11:10- 12:30. **Bacterial interactions (Chair: Pierre Cosson)**

9. Thierry Soldati: A TRAF-like E3 ubiquitin ligase orchestrates endolysosomal membrane damage repair and cell-autonomous immunity to *Mycobacterium marinum*.
10. Amber Ide: Exploring the functional link between Copine A and PatA, a Ca2+-ATPase pump, in Dictyostelium
11. Ramesh Rijal: Polyphosphate biology at the host-microbe interface
12. Otmane Lamrabet: *Dictyostelium discoideum* uses different mechanisms to kill Pseudomonas aeruginosa extracellularly and in phagosomes

Lunch: 1-2pm

2:00-3:20pm **Chemistry (Chair: Pierre Stallforth)**

13. Pierre Stallforth/Markus Günther: Natural products in bacteria-amoebae interactions
14. Robin Williams: Ketogenic diet-associated decanoic acid shows evolutionarily conserved mTORC1 inhibition
15. Falk Hillmann: Engineering polyketide production routes in *Dictyostelium discoideum* by exploiting native and synthetic hybrid enzymes
16. Chris West: Oxygen Sensors in *Dictyostelium* Development

Coffee 3:20-4:00pm

4:00-5:40 **Evolution/Ecology (Chair: Christina Schilde)**

17. Sandra Baldauf: Evolution of development in the aggregative multicellular amoeba, *Acrasis kona*
18. Sandrine Adiba: Adhesion and mechanics in social evolution
19. Tera Levin: Using genomes from *D. discoideum* sister species to reveal signatures of natural selection
20. Tsuyoshi Araki: Chemical communications between Dictyostelids and plant-parasitic nematodes
21. Pauline Schaap: Resolving the rationale of *Dictyostelium* signaling pathways: the power of the evolutionary comparative approach

Dinner: 6:30pm

8pm: Evening session, posters (even numbers present)

Tuesday 9th August

9:00-10:40. **Phagocytosis and beyond…(Chair: Thierry Soldati)**

22. James Vines: Some Wine With Your Meal? PIKfyve Drives Macropinosome/Phagosome Fusion to Aid Digestion
23. Sarah Körber: Convergence of Ras- and Rac-regulated formin pathways is pivotal for phagosome formation and particle uptake in *Dictyostelium*
24. Eunice Dominguez-Martin: The c-di-GMP synthase dgcA is required for proper function of *Dictyostelium* acidic-organelles
25. Pierre Cosson: Killing of bacteria in phagosomes: a biochemical and genetic dissection in *Dictyostelium discoideum*
26. Yoko Yamada: Autophagy and endosomal systems in *Dictyostelium* cell differentiation

Coffee 10:40-11:10

11:10- 12:30. **Pathogens/disease (Chair: Tera Levin)**

27. Aby Anand: Induction of membrane contact sites and manipulation of host lipid transfer proteins by pathogenic mycobacteria
28. Margaret Steele: Genetic control of predation resistance
29. Matthew Wielgat: Anti-biofilm compounds induced by Dictoystelid ameoba, *Polysphondylium pallidum*, degrade *Staphylococcus epidermidis* biofilms
30. Davide D'Amico: Are Discoidins evolutionary precursors of the galectin family of cytosolic lectins?

Afternoon: Excursions, with packed lunch

Dinner: 6:30 pm

8pm: Evening session, posters (odd numbers present)

Wednesday 10th August
9:00-10:40. **Gene regulation (Chair: Catherine Pears)**

31. Eric Greer: Epigenetics and multicellularity
32. Christian Hammann: Fractional chemical modification in the ribosomal RNA of *Dictyostelium discoideum* supports ribosome heterogeneity in Amoebozoa
33. Sabateeshan Mathavarajah: *Dictyostelium* PRP4K is required for macropinocytosis and multicellular development
34. Bart Edelbroek: Evolution of microRNAs in Amoebozoa
35. Fredrik Soderbom: Abundantly expressed class of noncoding RNAs conserved through the multicellular evolution of dictyostelid social amoebas

Coffee: 10:40-11:10

11.10- 12.30. **Cell Biology (Chair: Cynthia Damer)**
36. Annette Müller-Taubenberger: Centrosome positioning in migrating *Dictyostelium* cells
37. Marianne Grafe :Nuclear envelope dynamics during semi-closed mitosis
38. Holly Haver: Mechanistic insight into suppression of polyglutamine aggregation by Srcp
39. Jonas Kjellin: Presence and function of bacterial Rhs toxins in social amoebas

Lunch 1:00-2:00pm

2.00-3.40. **Actin and motility (Chair: Peter Van Haastert)**

40. Simona Buracco: Identification of a new actin polymerization function of the WRC independent of the VCA domain, but dependent on its polyproline domains
41. Adam Dowdell: Predicting novel migration behaviours
42. Tian Jin: How phagocytes acquired the capability of hunting and removing pathogens: lessons learned from chemotaxis and phagocytosis of *Dictyostelium discoideum*
43. Emily Hager: Observing cellular slime mold aggregation and signaling in transparent soil microcosms
44. Petra Fey: dictyBase (DCR) updates

Coffee: 3:40-16:20pm

16:20-17:50 **Chemotaxis 2 (Chair: Robert Insall)**

45. Christophe Anjard: *Dictyostelium* and *Acanthamoeba* present different motility response to hypoxia and oxygen gradients
46. Alan Kimmel: The *Dictyostelium* zinc finger protein TtpA regulates the co-ordinated stability of a group of mRNAs, through a common 3' UTR sequence
47. Peter van Haastert: From pseudopod to chemotaxis
48. Robert Kay/Josiah Lutton: The bubble wand model for macropinocytosis

Evening:
18:30 Pre-banquet drinks reception
19:00 Banquet
21:00 Ceilidh

Thursday 11th August: Breakfast and depart.

Speaker Abstracts

1. **Cortical actomyosin suppresses directed migration signaling networks**

Jonathan Kuhn: jkuhn14@jhmi.edu

Many modes of eukaryotic migration require cells to separate the newly polymerizing actin meshwork at the cell front from actomyosin-based contraction at the cell back. The location and configuration of cytoskeletal molecules are controlled by upstream signal transduction events. The terms, Signal Transduction and Cytoskeletal Excitable Networks, STEN and CEN, reflect the biochemically excitable properties displayed by molecules in each of these systems. Much is understood about how the STEN regulates the CEN in random and directed migration. To examine how changes in cytoskeletal mechanics and organization might feedback to influence STEN, we developed methods to rapidly and locally alter myosin activity and cortical actin organization in migrating vegetative and developed cells. First, we show that reducing myosin assembly leads to an increase in activated Ras and an increased sensitivity to chemotactic stimulus. Second, activation of cortical actin regulator RacE decreases STEN activation. Surprisingly, RacE activation leads to elevated actin polymerization, but this formation is relatively resistant to Arp 2/3 inhibition. Concomitantly, RacE activation causes a reduction in Ras activation and PtdIns(3,4,5)P3 levels. Taken together, our data suggest that cortical actomyosin suppresses cell front signaling molecules and suggests a novel mechanism for how cells can maintain front-back segregation during migration.

2. Establishing the coding properties of spiral waves

Hugh Ford: dmcbhfo@ucl.ac.uk

Hugh Ford, Angelika Manhart, Jonathan Chubb

Periodic cAMP waves regulate the transition to multicellularity during Dictyostelium development. This signalling is typically organised as a rotating spiral wave, which sets the dynamic properties of the long-range cAMP waves. The cAMP wave frequency steadily increases over time, coinciding with the transition to collective migration and aggregation, however the mechanisms underlying this change in frequency are poorly understood. To characterise these principles of cAMP signal coding and modulation, I have used the Flamindo2 cAMP reporter to track the signalling state and motion of large numbers of single cells both over the time and length scales of aggregation. We find that the spiral wave rotation rate and curvature increase over time, resulting in a decrease in the global wave period and speed, and the onset of aggregation. We test specific hypotheses for the increase in wave frequency: a) that cells become more excitable at high densities, b) that the change wave dynamics is a result of the spatial reorganisation of cells at the spiral core. Using experiments where we mechanically constrain the morphogenesis of the spiral core, we show that neither model fits the data. Instead, using the general theory of excitable media, we show that the excitability of the cells increases due to the increase in wave frequency, and that the wave frequency increases due to the increase in cell excitability. This finding suggests that the morphogenetic transition gradually unfolds due to coupling between the signalling state of cells and the patterns that result from signalling.

3. Ras Inhibitors Gate Chemoattractant Concentration Range for Chemotaxis in Eukaryotic Cells

Xuehua Xu: xxu@niaid.nih.gov

Eukaryotic cells use G protein coupled receptor (GPCR) to sense and move toward chemoattractant gradients through adaptation. Adaptive cells no longer respond to the present stimulus but remain sensitive to stronger stimuli. Thus, adaptation provides a fundamental strategy for eukaryotic cells to chemotax through a gradient with enormous concentration range. Ras activation is the first step of chemosensing signaling pathways that displays an transient activation behavior in both model organism Dictyostelium and mammalian neutrophils. Recently, we identified C2GAP1 and CAPRI in Dictyostelium and human neutrophil, respectively, that control both the GPCR-mediated adaption. More importantly, both Ras inhibitors regulates the sensitivity of the cells. These findings suggest an evolutionarily conserved molecular mechanism by which eukaryotic cell gates chemoattractant concentration range for chemotaxis.

4. CRISPR/Cas9-based genome-wide screening of Dictyostelium

Tetsuya Muramoto: tetsuya.muramoto@sci.toho-u.ac.jp

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Genome-wide screening is powerful method used to identify genes and pathways associated with a phenotype of interest. To address the inadequacies of conventional genetic screening approaches, we developed a highly efficient CRISPR/Cas9-based genome-wide screening system. A genome-wide library of 27,405 gRNAs and a kinase library of 4,582 gRNAs were compiled and mutant pools were generated. The resulting mutants were screened for defects in cell growth and more than 10 candidate genes were identified. Six of these were validated and five recreated mutants presented with growth abnormalities. Finally, the genes implicated in developmental defects were screened to identify the unknown genes associated with a phenotype of interest. These findings demonstrate the potential of the CRISPR/Cas9 system as an efficient genome-wide screening method.

5. The most important time in Dicty's life

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Satoshi Kuwana, Hidenori Hashimura, Satoshi Sawai
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During culmination of D. discoideum, cells undergo coordinated cellular movement and rearrangement to shape the final fruiting body anatomy. Cells reposition themselves from their original linear ordering along the antero-posterior axis to more convoluted three-dimensional layout with diploblastic-like radial symmetry. Time-lapse microscopic analysis of this dynamics is challenging due to the elongated tissue morphology encompassing large distances relative to the high speed of cell migration, thus we know surprisingly little about the dynamics - when and where cells move and in what way. Here we present our recent data obtained by light-sheet and confocal microscopy approach tailored to capture the hidden layers of this complex dynamics. We show that the initial stage of culmination is characterized by the appearance of a small cluster of ecmB expressing (PstB) cells at the tip region. The cluster appears to serve as a mold for the surrounding ecmA expressing (PstA) cells to polarize and rearrange in a collar-like pseudo-stratified (i.e. single) layer to curve the initial funnel-like cellulose-rich structure that become woven into a perfectly symmetric cellulose-enriched tube. The alignment of the PstA cells along the newly forming stalk tube is accompanied by marked localization of focal adhesion complex proteins - Paxillin, alpha-actinin, vinculin together with Apr2 and Myosin II. The elongation of the prestalk region occurs by intercalating rearrangement of the prestalk cells that converge along the stalk axis to form a single-layer. At the very edge of the intercalated layer, the PstA cells exhibit cell constriction, loss of cell polarity and rapid induction of the ecmB gene. The nascent PstB cells move in a reverse orientation thus completing the involution of the prestalk layer. These results suggest that folding cell layers as in gastrulation and their hallmarks i.e. cell intercalation, cell constriction and epithelial-to-mesenchymal like transition are inventions not singular to the metazoan lineages.

6. Excitable signalling drives a discontinuous developmental transition

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Elizabeth Westbrook, Vlatka Antolović, Tchern Lenn, Jonathan Chubb
University College London

Development can occur with discontinuous transitions between cell states. The regulation and functional implications of discontinuous transitions are not clear. We have defined a sharp "jump" in global gene expression during the development of Dictyostelium, corresponding to a discontinuity in cell states. Using live imaging of transcription, over millimetre length scales in the developmental niche, we show that this jump occurs at the transition between single cell and multicellular developmental states. Imaging signalling together with transcription reveals the jump coincides with the onset of collective cAMP signalling. During the transition, transcription of jump genes converts from sporadic to oscillatory behaviour. cAMP signalling is required for oscillatory but not sporadic transcription dynamics. Optogenetic activation of cAMP signalling is sufficient to induce jump gene transcription in pre-jump cells. Post-jump cells become resistant to stimuli promoting de-differentiation, suggesting the jump allows cells to "clear the slate" and commit to the next stage.

7. Evidence that the cAMP signalling system provides a coupled oscillator and allows developmental time to be encoded

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The transition from individual behaviour to coordinated group behaviour is a key feature in the evolution of multicellular developmental systems. Coordination is evident in cell movements, transcription and differentiation in space. Furthermore, cells are able to coordinate these behaviours in time in the absence of access to an external clock.

It has been shown theoretically that oscillatory signalling provides a mechanism to drive collective behaviour. In this, coupling of individual cellular oscillators through positive and negative feedback can allow populations of cells to synchronise their behaviour. Furthermore, the number of oscillations detected, or changes in the amplitude or frequency of oscillatory signal could encode temporal information.

Indeed, oscillatory cAMP signalling has been hypothesised to drive synchronised collective behaviour in the social amoeba D. discoideum, as well as temporal changes in transcription and cell behaviour seen during early development. Models suggest that periodic release and breakdown of cAMP is initiated in single cells, but transcriptional feedback loops act to increase levels of signal and help synchronise pulsatile cAMP synthesis and release across populations of cells. In turn, as the amplitude and frequency of cAMP pulses increase cells then further amplify this feedback through directed migration towards signalling centres.

One problem with these ideas, is that to date study of this system has been limited to visualising oscillations in cAMP, which lack the resolution to study the transition to collective behaviour at a single cell level. Furthermore, despite our knowledge of cAMP signalling relay components, the circuit dynamics that provide robust oscillations are yet to be fully understood.

One breakthrough, however, came from the observation that cells respond to cAMP oscillations through nucleocytoplasmic shuttling of the transcription factor GtaC, which acts as a positive regulator of developmental gene expression. We hypothesised that if GtaC encodes temporal information by providing positive feedback into cAMP signalling, then we should see GtaC shuttling at the onset of cAMP oscillations, or even before cAMP oscillations were visible.

Surprisingly, however, we found that GtaC is initially cytoplasmic and shuttling only begins several hours after cAMP oscillations can be detected, coinciding with increases in

synchronous cell movement. Indeed, GtaC mutant cells show cAMP oscillations, and their major defect is seen in inefficient cell migration.

We therefore hypothesised that another transcription factor(s) must exist that regulates early feedback required for the onset of collective behaviour. Consistent with this idea, we identified another transcription factor (Hbx5) that displays unsynchronised nucleocytoplasmic shuttling before population level cAMP oscillations can be detected, and then becomes synchronised as cAMP oscillations become detectable. Nucleocytoplasmic shuttling of Hbx5 can be induced by sub micro molar levels of cAMP and Hbx5 mutant cells display a complete failure to aggregate and little increase aggregative gene expression following starvation. Finally we find that Hbx5, like GtaC, shuttling is dependent on Erk2 activity which itself has been shown to oscillate, with the magnitude of oscillations increasing during development. Based on these findings, we propose a model in which initial stochastic oscillations in Hbx5 activity provide the initial feedback required for the onset of collective behaviour. Once collective oscillations have been established, the magnitude of Erk2 oscillations become sufficient to allow import and export of GtaC, thus ensuring that further feedback due to chemotaxis occurs at the appropriate developmental stage.

These studies provide evidence that D. discoideum behaves as a coupled oscillator, which allows the coordination of collective behaviour and developmental time to be encoded.

8. Information averaging facilitates symmetry breaking, lineage commitment and developmental robustness

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Many developmental systems are often predictable because they tightly regulated by molecular determinants of developmental signalling. We have found that this is exemplified by D. discoideum, where depends on the proportion of cells at different cell cycle positions. Pseudotime analysis of scRNA-seq data through a developmental time series taken at 2 hr intervals reveals three lineages of cells that can be reliably tracked from vegetative growth through to prestalk, prespore and ALC fates. Furthermore, we find that this is underpinned by an extensive suite of common markers of gene expression that can be viewed as a temporal signature shared by all cell types. One puzzling feature of this deterministic view of development, however, is that environmental perturbations would be expected to affect the level of molecular determinants of cell fate. Indeed the cell cycle is easily perturbed by environmental shifts in temperature or nutrition. Cell type proportioning is, however, remarkably robust in D. discoideum, like many developmental systems. Modelling and quantitative single cell measurements suggest this robustness is due to the integration of deterministic information about cell cycle position with an additional source of cell-cell heterogeneity. Analyses of scRNA-seq data reveal considerable stochastic gene expression variation in vegetative cells, which is controlled by epigenetic modifications. Stochastic and cell-cycle variation were found to be independently controlled and perturbation of each system results in relatively minor effects on development. However, when they are simultaneously disrupted there are severe defects in cell fate choice that cause development to fail. These data suggest the averaging of deterministic and stochastic signals can provide a simple mechanism for the evolution of robustness when there are constraints on increasing signalling fidelity or reducing exposure to population level environmental perturbations.

9. A TRAF-like E3 ubiquitin ligase orchestrates endolysosomal membrane damage repair and cell-autonomous immunity to *Mycobacterium marinum.*

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Abstract
We use Dictyostelium discoideum as a host cell for the pathogenic bacterium *Mycobacterium marinum*, a close relative of Mycobacterium tuberculosis to study the molecular mechanisms of host-pathogen interactions during infection. Following uptake, *M. marinum* inhibits host defense mechanisms such as autophagy and lysosomal-dependent degradation, to establish a permissive niche with endosomal features, the mycobacteria-containing vacuole (MCV), where it replicates. *M. marinum* damages the MCV membrane already 1 hour post infection (hpi). Although these damages ultimately are required for the pathogen translocation to the host cytosol and further dissemination, they also allow the detection of the bacteria, its labelling with autophagy "eat-me" signals such as K63-linked polyubiquitin chains and restriction of the pathogen intracellular proliferation. We identified the *D. discoide*um E3 ubiquitin ligase TrafE and show that GFP-TrafE is recruited to the *M. marinum* MCV after 1 hpi in a damage-dependent manner. In the absence of TrafE, the number of MCV / bacteria positive for K63 Ub as well as the intracellular growth of the pathogen are drastically reduced, and *M. marinum* is highly toxic for trafE-KO mutant cells. We also show that GFP-TrafE is recruited to endo-lysosomes upon LLOMe-induced sterile damage and that trafE-KO cells fail to repair damaged acidic compartments as indicated by their failure to reacidify. Finally, lack of TrafE results in abnormal autophagy and ESCRT responses to sterile damage and membrane tension decrease alone is enough for GFP-TrafE recruitment to endo-lysosomal compartments. Altogether, our data suggest that TrafE is involved in the detection of damage to intracellular compartments and is required for *M. marinum* restriction and endo-lysosomal homeostasis.

10. Exploring the functional link between Copine A and PatA, a Ca2+-ATPase pump, in Dictyostelium

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Copines (Cpn) are a family of cytosolic and nuclear proteins that associate with membranes in a calcium-dependent manner and are found in many eukaryotic organisms. Dictyostelium discoideum has six known copine genes, cpnA-cpnF, and we have focused our studies on CpnA. CpnA strongly binds to phosphatidylserine (PS) and translocates from the cytosol to the plasma membrane in response to a rise in calcium. Cells lacking cpnA (cpnA-) exhibit many mutant phenotypes including defects in development, cytokinesis, membrane trafficking, chemotaxis, and adhesion. Previous research showed cpnA- cells have increased adhesion to substrate, fluorescent beads, and bacteria and this was due to increased PS exposure on the outer leaflet of the plasma membrane. Using fura-2 dextran and FITC-lactadherin, we found that cpnA- cells have increased intracellular calcium concentrations and increased PS exposure compared to wild-type cells. Chelating calcium with EGTA decreased intracellular calcium concentrations and decreased PS exposure in both cpnA- and wild-type cells. Starving wild-type and cpnA- cells for six hours showed high levels of intracellular calcium but low PS exposure in both cell lines, which could be due to decreased TMEM16 expression, a calcium-dependent phospholipid translocase. The increased intracellular calcium defect in cpnA- cells could be due to a defect in a calcium pump. Previous research showed cpnA- cells made very large contractile vacuoles that could not be exocytosed properly compared to wild-type cells. In Dictyostelium, the Ca2+-ATPase pump, PatA, localizes to the contractile vacuole membrane and is involved in regulating calcium homeostasis. Using a patA antisense plasmid, we knocked down the expression of PatA in wild-type cells and found that the patAKD cells made large contractile vacuoles and had high PS exposure similar to cpnA- cells. Overall, this suggests that CpnA may function as an activator of the PatA Ca2+-ATPase pump.

11. Polyphosphate biology at the host-microbe interface

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Polyphosphate (polyP) is a linear polymer of orthophosphates linked by high-energy phosphoanhydride bonds and is secreted by bacteria and eukaryotes. Decreased polyP levels in pathogens, such as Mycobacterium tuberculosis and Salmonella enterica, are associated with reduced pathogenicity. How bacterial polyP affects pathogenicity is unknown. We found that Dictyostelium cells accumulate extracellular polyP at high cell densities, and the extracellular polyP inhibits proliferation, inhibits phagosome acidification and lysosome activity, and causes cells to retain ingested nutrients by inhibiting excretion. This may allow cells to prepare for starvation by potentiating the formation of large cells with large amounts of stored nutrients.

Possibly as a result of these effects on phagosome maturation, polyP potentiates the survival of phagocytosed Escherichia coli or M. smegmatis in Dictyostelium. This effect of polyP was also observed in human macrophages. Reducing the expression of polyphosphate kinase 1 in M. smegmatis reduces the accumulation of extracellular polyP and reduces M. smegmatis survival in Dictyostelium or macrophages. Dictyostelium cells lacking the G protein-coupled polyP receptor GrlD, or proteins involved in the mTOR signaling pathway and some other signaling pathways, do not decrease their killing of phagocytosed bacteria in the presence of polyP.

Pathogens such as M. tuberculosis secrete extracellular polyP, which drives an anti-inflammatory M2 activation of macrophages and impairs the expression of MHC class II molecules to hinder the adaptive immune response. Treatment of human macrophages with recombinant yeast exopolyphosphatase or pharmacological inhibitors of proteins identified in the Dictyostelium assays potentiate autophagy marker LC3 expression, potentiate the pro-inflammatory M1 activation of macrophages, and reduce the survival of M. tuberculosis in macrophages. Together, these results suggest that 1) Dictyostelium cells use polyP as a signal to sense local cell density, and at high cell densities, reduce the digestion of, and retain, ingested nutrients (including some live bacteria) in anticipation of starvation, 2) that for unknown reasons a similar polyP inhibiting bacterial killing mechanism exists in human macrophages, and 3) that pathogens such as M. tuberculosis may secrete polyP to mimic this effect and survive in human macrophages.

12. *Dictyostelium discoideum* uses different mechanisms to kill *Pseudomonas aeruginosa* extracellularly and in phagosomes

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Neutrophils can kill bacteria in phagosomes after ingesting them (intracellular killing), or without ingesting them by secreting bacteriolytic molecules in their close vicinity (extracellular killing). The contribution of different antibacterial to intracellular and extracellular killing remains unclear. We used Dictyostelium discoideum as a model host to determine the involvement of different antibacterial mechanisms in intracellular and extracellular killing.
When D. discoideum encounters a Pseudomonas aeruginosa bacterium, it can either ingest it or not. Remarkably, a large proportion of bacteria that are not ingested die a few minutes after being in contact with D. discoideum. These observations indicate that D. discoideum can kill P. aeruginosa intracellularly and extracellularly.
In this study, we used a collection of D. discoideum mutants to identify the molecular mechanisms involved in the extracellular and intracellular killing of P. aeruginosa. Our results indicate that the intracellular and the extracellular killing of P. aeruginosa by D. discoideum mobilize different cellular mechanisms.

13. Natural products in bacteria-amoebae interactions

Pierre Stallforth/Markus Günther

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During the development of Dictyostelium discoideum, the production of a large set of polyketide synthases is activated.[1]. Previous research, however, mostly focused on two polyketide synthase genes, namely stlA and stlB [2,3], involved in the production of the secondary metabolites DIF-1 and MPDB. Thus, virtually nothing is known about the remaining pks genes and their products. We isolated and characterized the pks5-derived polyketide dictyodene, a deep yellow pigment, which prevents premature hatching of amoebal spores. In our study, we combined complementary molecular biology approaches, including CRISPR-Cas9 and promoter studies and generated polyketide synthase inactivation and overproduction strains.

14. Ketogenic diet-associated decanoic acid shows evolutionarily conserved mTORC1 inhibition

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D. discoideum provides an excellent model for biomedical research, due a combination of factors including evolutionary conservation of proteins link to diseases in humans and a range of molecular methods allowing innovative research. One example of this research relates to identifying new treatments for epilepsy, where discoveries in Dictyostelium have led to the development of a new dietary treatment, 'K.Vita', that has been validated in clinical trials. These 'ketogenic' dietary treatments are generally related to high fat and low carbohydrate intake, where reduced glucose and insulin conditions are thought to reduce activity of the mechanistic target of rapamycin complex 1 (mTORC1) signalling pathway, leading to a range of positive medical and health-related effects. Here, we investigated a key component of the medium chain triglyceride (MCT) ketogenic diet, decanoic acid, in the regulation of mTORC1 signalling using Dictyostelium. We showed that decanoic acid decreased mTORC1 activity, measured by phosphorylation of 4E-BP1, independent of glucose and insulin levels. To describe this mechanism, we showed that decanoic acid functioned through a ubiquitin regulatory X (UBX) domain-containing protein (UBXD18) to inhibit p97 activity and reduce mTORC1 activity. We then translated this effect to a rat hippocampal slice model and patient-derived astrocytes with tuberous sclerosis complex (TSC) mutations to show that decanoic acid also decreased mTORC1 activity, in the absence of insulin and under high glucose conditions. Our data therefore identified a new and evolutionarily conserved effect of decanoic acid in down-regulating mTORC1 signalling, with potential relevance to a wide range of medical treatments.

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15. Engineering polyketide production routes in Dictyostelium discoideum by exploiting native and synthetic hybrid enzymes

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Aromatic polyketides are natural polyphenolic compounds having a broad spectrum of pharmacological activities. Genome sequencing continuously expands the number of novel polyketide synthases (PKSs) in all kingdoms of life but often without a link to actual molecules, their biological activity or therapeutic potential. Heterologous expression of the producing enzymes in bacteria such as Escherichia coli or in the yeast Saccharomyces cerevisiae presents a widely known alternative to produce these molecules, but the lack of accessory secondary metabolic pathways in these model organisms often remains a limiting factor.
Social amoebae are intrinsic producers of such polyketides and harbor a large reservoir of PKS genes, two of them encoding unique types of natural hybrid enzymes of type I fatty acid synthases and type III PKSs. Here, we have exploited the social Dictyostelium discoideum as a chassis for the production of aromatic polyketides by expressing its native and cognate plant PKS genes. Extrachromosomal expression of natural hybrids led to the production of phlorocaprophenone and methyl-olivetol. Further genetic engineering using various plant polyketide synthases and a eukaryotic multi-gene expression tool led to the formation of naringenin, resveratrol, and olivetolic acid (OA). The latter acts as the central intermediate in the cannabinoid biosynthesis pathway. To further facilitate OA synthesis, we engineered a synthetic amoeba/plant inter-kingdom hybrid enzyme which produced OA from primary metabolites in only two enzymatic steps, providing a shortcut in the plant cannabinoid pathway. As the amoebae could also be successfully cultivated in high cell densities in bioreactors at the hundred liter scale, we are currently evaluating D. discoideum as a novel microbial host system for the production of polyketides.

16. Oxygen Sensors in Dictyostelium Development

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Dictyostelium exhibits characteristic responses to oxygen at its single cell and multicellular stages. We have found 3 non-heme dioxygenases that contribute to the O2-dependent control of culmination. PhyA, a presumptive ortholog of human PHD2 that modifies of HIFalpha in humans, instead modifies the Skp1 subunit of the SCF family of E3 ubiquitin ligases and their activity. Our proteomics analysis of the Skp1 interactome identified dozens of putative F-box proteins expected to serve as substrate receptors, and whose binding to Skp1 depends on Skp1 hydroxylation. Further interactome studies of one substrate receptor, the F-box protein FbxWD, revealed Vwa1, a vault protein inter-alpha-trypsin (VIT) and von Willebrand Type-A (vWA) domain containing protein with homology to a very poorly understood human homolog associated with tumor suppression and schizophrenia. A functional codependence was revealed by finding that inhibition of terminal differentiation by FbxWD overexpression depended on intact Vwa1. The importance of Vwa1 was indicated by dominant negative effects of overexpression of its discrete domains, that also depended on Vwa1 – indicating an effect on its own activity. Examination of another F-box protein, the jumonji C-protein JcdI, revealed that PhyA negatively regulates its abundance in cells. JcdI is highly conserved with the human lysyl hydroxylase JmjD6, which has been implicated in epigenetic regulation, RNA splicing and oncogenesis. Recombinant JcdI, also a non-heme dioxygenase, was found to have a high Km (O2) of 22%, and its overexpression reduced the O2-threshold for culmination as for PhyA. Another non-heme dioxygenase, an N-terminal protein cysteine oxidase (ADO), has been implicated in N-end rule dependent regulation of protein stability in plants and animals. ADO also has a high Km (O2), and its disruption increased the O2-threshold for culmination as for phyA-KO. These findings suggest that Dictyostelium utilizes parallel modes of O2-sensing to coordinate separate cellular processes of culmination.

17. Evolution of development in the aggregative multicellular amoeba, *Acrasis kona*

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The life cycle of the acrasid amoebae resembles that of dictyostelids, despite being nearly as distantly related as any two eukaryotes can be (suprakingdoms Excavata and Amorphea, respectively). We have sequenced the genome and three developmental transcriptomes from Acrasis kona and find that the genome is rich in novel genes, multigene families and genes acquired by horizontal transfer. Development in A. kona appears to be molecularly much simpler than in Dictyostelium discoideum, involving substantially increased expression of only 449 genes which is nearly 5-fold fewer than in the dictyostelid. However, a not insubstantial amount of this difference appears to reflect the fact that, unlike developing D. discoideum, developing A. kona does not appear to be starving. The two amoebae nonetheless show strong and extensive similarity in developmental signalling and, potentially, extracellular matrix construction. This suggests either a remarkable evolutionary convergence or an early origin of these pathways in eukaryotes.

18. Adhesion and mechanics in social evolution

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The social amoeba Dictyostelium discoideum commonly forms chimeric fruiting bodies. Genetic variants that produce a higher proportion of spores are predicted to undercut multicellular organization unless cooperators assort positively. Cell adhesion is considered a primary factor driving such assortment, influencing the capacity of cells to interact preferentially with those having the same social strategy. However, evolution of adhesion has not been experimentally connected to changes in social performance.
In this study, we modified by experimental evolution the efficiency of individual cells in attaching to a surface. Surprisingly, evolution appears to have produced social cooperators irrespective of whether stronger or weaker adhesion was selected.
Quantification of reproductive success, cell-cell adhesion and developmental patterns, however, revealed two distinct social behaviours, as captured when the classical metric for social success is generalized by considering clonal spore production.
Our work shows that cell mechanical interactions can constrain evolution of development and sociality in chimeras, and that elucidation of proximate mechanisms is necessary in order to understand the ultimate emergence of multicellular organization.

19. Using genomes from *D. discoi*deum sister species to reveal signatures of natural selection

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Evolutionary genomic analyses of conservation and selection can yield important insights into protein function. Because these approaches analyze genetic variation among different strains or species, they require genomes that are different enough that there is genetic variation to observe, but not so different that the genomic record has been repeatedly overwritten by mutation. In Dictyostelium, these evolutionary analyses have been difficult because many D. discoideum strains are extremely genetically similar, while the closest well-sequenced relatives such as purpureum are too diverged. To address this gap, we are performing whole genome sequencing on a suite of diverged D. discoideum strains and sister species and generating hybrid de novo assemblies from both short-read Illumina and long-read Nanopore data. So far, we have successfully assembled 4 Dictyostelium strains (2 discoideum strains from Costa Rica and Mexico, 1 citrinum from Kansas, and 1 intermedium from Indonesia). Using these assembled genomes, we plan to analyze the evolution of putative immune defense genes of Dicty, as this category of genes often evolves rapidly in other taxa. Once complete, this set of assembled genomes will be broadly useful for investigating signatures of selection, speciation, and gene family expansions within the Dictyostelium clade. We present the results piloting this approach for initial candidate genes such as tirA, which has been previously implicated in amoeba-bacteria interactions. Through this work, we are constructing a dataset of diverged discoideum relatives ideal for evolutionary analyses.

20. Chemical communications between Dictyostelids and plant-parasitic nematodes

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The soil nematodes, free-living nematodes and plant-parasitic nematodes, coexist with Dictyostelids in soil, and both are the constituents of soil biota. Free-living nematodes, such as Caenorhabditis elegans, are thought to be the competitors for food, have a relationship of prey-predator with Dictyostelids and possibly function as spreaders of spores (1). In contrast, the relationships between plant-parasitic nematodes and Dictyostelids are not well-understood.
We have found that Dictyostelids can repel root-knot nematodes, Meloidogyne incogninta and M. hapla, and their repellent behaviours are reacting to chemical compounds released by fruiting bodies of Dictyostelids in all four groups (2). These chemical extracts can repel other group of plant-parasitic nematodes, lesion nematodes Pratylenchus coffeae and P. penetrans. Under laboratory conditions, the fruiting body extracts show repellent activity strong enough to shield plant roots from infection of plant-parasitic nematodes. A series of chemical purification steps, HPLC and GC-MS analysis revealed that a substance with molecular mass 208 and molecular formula C8H16O6 has repellent activity, and we further identified this substance as non-reducing sugar, Ethyl-alpha-D-glucoside (alphaEG).
These results suggest that there exist chemical communications between Dictyostelids and plant-parasitic nematodes and Dictyostelids-released compounds may be of use as control agents for plant-parasitic nematodes.

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21. Resolving the rationale of Dictyostelium signaling pathways: the power of the evolutionary comparative approach

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My team uses both single organism and evolutionary comparative approaches to understand the signaling pathways that regulate Dictyostelium development. In this presentation I will summarize the results of comparative cell-type specific and developmental transcriptomics and comparative transcription factor gene family analysis that highlighted evolutionary change in transcription factors that may have given rise to novel cell types. One such example was a gene duplication in the family of cudA-like transcription factors that only occurred in group 4, which contains D.discoideum. One replicate, cdl1a, showed signatures of positive selection, which are indicative of functional innovation. Deletion of cdl1a resulted in fruiting bodies with sagging spore heads and loss of cup-specific gene expression and cup cells, which are only present in group 4. Deletion of cdl1b cause no phenotypic alterations, but a cdl1b^{-}cdl1a^{-} double knock-out was severely defective in stalk formation, suggesting an ancestral role for cdl1 in stalk formation. This was confirmed in P.violaceum, a close sister species to group 4, where deletion of cdl1 also caused severe stalk defects. While duplication of gene regulatory proteins is often thought to cause cell type divergence, this is the first proven example of a duplicated transcription factor being required for the evolution of a novel cell type.

22. Some Wine With Your Meal? PIKfyve Drives Macropinosome/Phagosome Fusion to Aid Digestion

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PIKfyve, a PI-5 kinase, is essential for efficient phagosome maturation, and its inactivation perturbs luminal acidification and proteolysis (Buckley et al., 2019). The mechanisms through which PIKfyve regulates these functions, and the dynamics of its product, PI(3,5)P2, remain poorly understood, however. Here we show, using a novel probe and the model phagocyte Dictyostelium, that PIKfyve drives delivery of PI(3,5)P2 and Rab7-positive compartments to early phagosomal membranes. Using pulse chase assays, we identify these compartments as early macropinosomes, suggesting, for the first time, phagosome-macropinosome fusion events may be important for maturation. Furthermore, inactivation of PIKfyve perturbs the delivery of these Rab7 positive macropinosomes, reducing the level of phagosome-macropinosome mixing, Rab7 acquisition, and consequently lysosomal enzyme delivery. PIKfyve, or its product PI(3,5)P2, therefore regulates fusion between Rab7 positive phagosomes and macropinosomes, without which efficient phagosomal maturation is not possible.

23. Convergence of Ras- and Rac-regulated formin pathways is pivotal for phagosome formation and particle uptake in Dictyostelium

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Macroendocytosis, comprising phagocytosis and macropinocytosis, are actin-driven processes regulated by small GTPases that depend on the dynamic reorganization of the membrane that protrudes and internalizes extracellular material by cup-shaped structures. To effectively capture, enwrap, and internalize their targets, these cups are arranged into a peripheral ring or ruffle of protruding actin sheets emerging from an actin-rich, non-protrusive zone at its base. Despite extensive knowledge of the mechanism driving actin assembly of the branched network at the protrusive cup edge, which is initiated by the actin-related protein (Arp) 2/3 complex downstream of Rac signaling, our understanding of actin assembly in the base is still incomplete. In the *Dictyostelium* model system, the Ras-regulated formin ForG was previously shown to specifically contribute to actin assembly at the cup base. Loss of ForG is associated with a strongly impaired macroendocytosis and a 50% reduction of F-actin content at the base of phagocytic cups, in turn indicating the presence of additional factors that specifically contribute to actin formation at the base. Here, we show that ForG synergizes with the Rac-regulated formin ForB to form the bulk of linear filaments at the cup base. Consistently, combined loss of both formins virtually abolishes cup formation and leads to severe defects of macroendocytosis, emphasizing the relevance of converging Ras- and Rac-regulated formin pathways in assembly of linear filaments in the cup base, which apparently provide mechanical support to the entire structure. Most intriguingly, we finally show a prominent localization of ForB to the distal site of a phagosome right after cup closure, coinciding with the appearance of an actin-rich comet tail and rocketing of the phagosome deeper into the cell. This function is conferred specifically by ForB since its loss or overexpression directly correlates with travelled distance and speed of rocketing phagosomes, whereas no such activity could be observed for ForG.

24. The c-di-GMP synthase dgcA is required for proper function of Dictyostelium acidic-organelles

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Previous work from the P. Schaap group has demonstrated that cyclic-di-guanosine-monophosphate (c-di-GMP) synthesized by dgcA is essential for stalk differentiation during Dictyostelium discoideum development1, through regulating a stalk-specific Protein Kinase A (PKA)-dependent transcriptional program2. In addition to its role in transcription regulation, c-di-GMP may play a role in autophagy, since it has been reported that it synergizes with DIF-1 during autophagic cell death induction of starved cells in monolayers3. In agreement with this observation, recent reports have suggested a connection between cyclic dinucleotide signaling (CDN) and autophagy-related processes in animal cells. A direct role of cGAS, the animal cyclic-GMP-AMP synthase, regulating the autophagy protein Beclin has been proposed4; while the animal cyclic dinucleotide receptor, STING, has been reported to be involved in various autophagy-related processes5–9. Interestingly, regulation of the autophagy-related processes may be the primordial and more conserved role of CDN signaling, since the inflammatory response is only conserved in mammalian organisms5,7,8,10–12

Based on these previously described associations between CDNs and autophagy, we addressed the relationship between autophagy and c-di-GMP in vegetative Dictyostelium cells and found that dgcA is required not only for autophagy degradation, but for proper function of the acidic-organelles network of this organism, comprised of the endolysosomal system and the contractile vacuole. dgcA is required for the expression of genes related to the hyperosmotic stress response, the actin cytoskeleton and to lysosomal function in vegetative axenic cells growing in liquid media and its lack, causes an increase of acidic-organelle pH, hampering the degradation of lysosomal cargoes. In addition, cells lacking dgcA present abnormal contractile vacuole morphology and compared to the WT, are less able to survive changes of osmolarity. In agreement with these observed phenotypes, we found that c-di-GMP intracellular concentration was regulated by changes in media osmolarity and by nutritional cues. In Dictyostelium, c-di-GMP may act as a messenger molecule that allows acidic-organelles adaptation to osmolarity or nutritional status variations. Currently, we are exploring the regulation of dgcA, and we have found that the N-terminal portion of dgcA is required to regulate its activity and that this protein may associate to membranes depending on its activation status, similarly to mammalian cGAS.

Based on our observations and previous research in metazoans, we propose that CDN signaling may have emerged in ancestral eukaryotes as signaling molecules able to adjust the function of its acidic-organelles to changes in nutritional cues, such as the presence of engulfed bacteria; and during eukaryote evolution, this pathway gained complexity, in Dictyostelium as part of the response to nutritional status, osmotic balance and during its developmental cycle; while in animal cells, it shaped innate immune responses.

25. Killing of bacteria in phagosomes: a biochemical and genetic dissection in Dictyostelium discoideum

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The general aim of our laboratory is to understand how phagocytic cells ingest and kill microorganisms. More specifically we want to establish a complete list of molecular mechanisms allowing killing of bacteria in phagosomes, to quantify their relative importance, and to determine which mechanisms kill different bacteria. We use Dictyostelium discoideum as a model phagocytic cell. In this model system, we combine biochemical methods (characterization of D. discoideum bacteriolytic molecules in vitro) and genetic analysis (selective gene inactivation in D. discoideum cells).
We have identified in vitro several new antibacterial proteins present in D. discoideum and verified that they are required for optimal killing in D. discoideum phagosomes. Our observations indicate that D. discoideum uses largely different molecular mechanisms to kill different species of bacteria.

26. Autophagy and endosomal systems in Dictyostelium cell differentiation

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The fruiting body of D. discoideum is composed of spores and three supporting cell types. In our study of signalling pathways that control spore formation, we isolated the autophagy gene, atg7, a gene of unknown function, knkA, and the phosphatidylinositol-3-phosphate 5-kinase, pikfyve, as genes required for formation of viable spores.

The atg7- and other autophagy mutants as atg5- and atg9- were specifically defective in cAMP induction of prespore genes. They failed to form viable spores but showed extensive differentiation of stalk-like cells.

The knkA- mutant also showed reduced in cAMP induction of prespore genes and did not form viable spores. In migrating slugs of the knkA-, prespore cells transdifferentiated into vacuolated basal disc cells at a high rate. Bcas3 isolated as an interacting protein of KnkA is shown to have similarities to atg18, a PROPPIN that binds PI3P and is involved in phagophore formation. Concordantly KnkA and Bcas3 colocalised at phagophore assembly sites and were required for full autophagy activity, indicating that they are autophagy regulators.

The pikfyve- cells initially differentiated into prespore cells, but at the time of spore maturation, the prespore vesicles with spore wall components fused to form a vacuole instead of being exocytosed. Vacuolation was accompanied with the expression of the basal disc marker genes.

The results altogether indicate functions of autophagy and endosomal regulation in cell fate choice of Dictyostelium. High autophagy activity favours spore formation, in marked contrast to its so far suggested link to stalk cell differentiation.

27. Induction of membrane contact sites and manipulation of host lipid transfer proteins by pathogenic mycobacteria

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Tuberculosis, caused by Mycobacterium tuberculosis (Mtb), is the second leading cause of death caused from a single infectious agent. Mtb is able to remodel the host lipid metabolic pathways to create an optimal niche for its survival and replication. In particular, Mtb has perfected many ways to utilize host fatty acids and sterols as carbon and energy source to support its intracellular persistence. Our key objective is to decipher the molecular mechanisms underlying host lipid acquisition by mycobacteria.

An emerging strategy of bacterial pathogens is the induction of membrane contact sites (MCSs) between biogenic host organelles and bacteria-containing vacuoles for lipid exchange. MCSs are typically enriched in lipid biosynthetic enzymes and transport machinery, notably cytosolic lipid transfer proteins (LTPs) that enable lipids to reach their destination independent of vesicular trafficking. Interestingly, in the attractive infection model, the Dictyostelium discoideum/Mycobacterium marinum system, LTPs from the oxysterol binding protein (OSBP) family were found abundantly in the proteome of isolated Mycobacterium-containing vacuoles (MCVs). This prompted us to investigate the subcellular localization of OSBP8 which is phylogenetically close to human OSBPs. Whereas OSBP8 is cytosolic and localizes at the Golgi apparatus and at the perinuclear ER in non-infected cells, it is recruited to the vicinity of the MCV as well as to cytosolic mycobacteria during infection. Strikingly, lattice light sheet microscopy and expansion microscopy revealed that pathogenic mycobacteria induce the formation of MCS between the MCV and the ER and recruit OSBP8 to these sites. Intriguingly, OSBP8 is not mobilized by non-pathogenic mycobacteria that lack the type VII secretion system ESX-1 or ESAT-6/CFP-10, respectively, indicating that bacterial effector proteins that are secreted via ESAT-6 pores might be involved in recruiting OSBP8.

Members of the OSBP family act as so-called lipid exchangers and have been shown to counter transport sterols in exchange for PI4P. Interestingly, PI4P accumulates at the MCV and is essential for OSBP8 mobilization. Consequently, in analogy to its human homologues, we postulate that OSBP8 mediates sterol/PI4P counter transport at MCS and might thus deliver sterols from the ER to the MCV providing the bacteria with sterols. Interestingly, mycobacteria growth is accelerated in OSBP8 KOs in which the distribution of sterols and PI4P is completely disrupted. We hypothesize that the block in ER-MCV lipid transport is compensated by an accumulation of sterols in endosomes that might lead to impaired xenophagy and pathogen degradation, respectively. We are now investigating the mode of action of OSBP8 and are identifying its lipid ligands with the help of purified proteins and lipid binding assays. In the future, we will apply photoactivatable probes to monitor sterol flows from the host to the pathogen.

28. Genetic control of predation resistance

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In nature, Dictyostelium discoideum is both a predator of bacteria and a host for bacterial pathogens. Many bacteria form transient associations with D. discoideum in soil. Interactions with D. discoideum may drive pathogen evolution by favoring adaptations that allow bacteria to survive phagocytosis and benefit from association with their would-be predator. To identify such adaptations, we examined 13 Pseudomonas strains isolated from wild D. discoideum clones. We identified three distantly related strains that are resistant to predation and capable of infecting the sorus, one of which feeds on amoebae. Using microscopy and an antibiotic protection assay, we determined that these infections are extracellular. To identify genes that contribute to predation resistance, we screened over 2,000 transposon mutants from each species for loss of resistance. We found 149 genes that are important for predation resistance, including genes involved in transcriptional regulation, signal transduction, cell wall and membrane biogenesis, outer membrane vesicle production, secondary metabolite biosynthesis, and putative toxins. Many mutants could be rescued from predation through co-culture with the wild type strain, suggesting that predation resistance can be a social behavior. Though most predation resistance genes differ between strains, gacA, a response regulator that controls production of many secondary metabolites, is required for predation resistance in all 3 species, indicating that Pseudomonas strains use both conserved and lineage-specific mechanisms to escape predation by D. discoideum.

29. Anti-biofilm compounds induced by Dictystelid ameoba, Polysphondylium pallidum, degrade Staphylococcus epidermidis biofilms

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Biofilm-related infections of Staphylococcus epidermidis are considered a leading cause of nosocomial sepsis and are the most frequently isolated organism from hospital-acquired infections. Due to the protective effect of biofilm extracellular polymeric substances, biofilm-forming bacteria avoid immune recognition and clearance in chronic wounds and tissue- or device-related infections. We have recently demonstrated that Dictyostelids, including Polysphondylium pallidum (El Salvador), can efficiently feed on biofilms of multiple bacterial species, including S. epidermidis. Given these observations, we sought to identify compounds potentially produced by Dictyostelid (Polysphondylium pallidum) consumption of S. epidermidis biofilms that are responsible for anti-biofilm activity. We hypothesized that Dictyostelid predation of S. epidermidis induces the production of anti-biofilm compounds. A chromatography-based isolation approach was developed to identify compounds potentially responsible for the anti-biofilm activity. The application of these compounds onto S. epidermidis biofilms caused a near-complete removal of biofilms within two hours. The present findings introduce new methods for eradicating established biofilm communities and highlight the therapeutic potential of dictyostelids in antimicrobial research.

30. Are Discoidins evolutionary precursors of the galectin family of cytosolic lectins?

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Glycans play a fundamental role in host-pathogen interactions because lectin-sugar bindings are the first molecular encounters when host and pathogen meet. Galectins are a family of lectins found both in the cytosol and secreted in an unconventional manner. They are widely expressed in metazoa have affinities for β-galactosides and are active in both self- and non-self-glycoconjugate recognition. Our model system, the social amoeba *Dictyostelium* discoideum expresses four canonical discoidins (Dsc), lectins that share molecular, biological characteristics and localization with galectins. Upon infection with Mycobacterium marinum, Dsc-GFP foci were observed in the vicinity of the bacterium. Our current work aims at defining a possible role in infection and to determine whether Dsc recognise self-glycans exposed by pathogen-induced host membrane rupture, or bacterial surface glycosylations made accessible after vacuole damage. Experiments with various sterile membrane damage agents showed no Dsc-GFP foci formation, thus ruling out recognition of host glycosylations. Alternatively, we performed in vitro binding assays using chemically-labelled Dsc isoforms, various bacteria and quantified binding by FACS. Preliminary results confirmed also by immunofluorescence indicate that both Dsc I and II can decorate bacteria and mammalian cells, but not D. discoideum amoebae, suggesting a role in non-self-recognition. In parallel, we aim to identify with a glycoarray approach all possible ligands for Dsc I and II corresponding to a library of known bacterial surface glycosylations. We also plan to engineer recombinant Dsc I and II with non-functional carbohydrate recognition domains to validate the glycoarray findings and monitor the impact of the mutations during infection with M. marinum.

31. Epigenetics and multicellularity

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The evolution of multicellularity is a critical event that remains incompletely understood. We use the social amoeba, Dictyostelium discoideum, one of the rare organisms that readily transits back and forth between both unicellular and multicellular stages, to examine the role of epigenetics in regulating multicellularity. While transitioning to multicellular states, patterns of H3K4 methylation and H3K27 acetylation significantly change. By combining transcriptomics, epigenomics, chromatin accessibility, and orthologous gene analyses with other unicellular and multicellular organisms, we identify 52 conserved genes, which are specifically accessible and expressed during multicellular states. We validated that four of these genes, including the H3K27 deacetylase hdaD, are necessary and that an SMC-like gene, smcl1, is sufficient for multicellularity in Dictyostelium. These results highlight the importance of epigenetics in reorganizing chromatin architecture to facilitate multicellularity in Dictyostelium discoideum and raise exciting possibilities about the role of epigenetics in the evolution of multicellularity more broadly.

32. Fractional chemical modification in the ribosomal RNA of Dictyostelium discoideum supports ribosome heterogeneity in Amoebozoa

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The two most abundant chemical modification of ribosomal RNA (rRNA) are 2′-O-methylation (2′-O-Me) and pseudouridylation (Ψ). The former is introduced sequence specifically by box C/D small nucleolar ribonucleoprotein (snoRNPs) complexes, while the latter is guided and performed by box H/ACA snoRNPs. The landscapes of both modifications were previously unknown for Dictyostelium discoideum and most data on these chemical modifications and its impact on RNA folding and stability are derived from organisms of the Opisthokonta supergroup. A recent study by the Kellner lab established the presence of both modifications in RNA molecules isolated from the amoeba (1).
To identify 2′-O-Me and Ψ in the sequence of the rRNAs of D. discoideum, we employed the next generation sequencing techniques RiboMeth-seq and HydraPsiSeq in axenic growth and development. Using RiboMeth-seq, we found 49 positions in the 17S and 26S rRNA 2′-O-methylated (2). Several of these nucleotides are substoichiometrically modified, with one displaying dynamic modification levels during development. Using bioinformatics and RNA-seq data, we identified 30 novel box C/D snoRNAs, many of which are differentially expressed during the multicellular development of the amoeba. For most 2′-O-methylated positions, a guiding box C/D snoRNA could be identified, allowing to determine idiosyncratic features of the box C/D snoRNA/rRNA interactions in the amoeba (2).
As for Ψ, we identified 66 positions in the 5.8S, 17S, and 26S rRNAs, with 31 of these nucleotides being differentially modified at the eight-hour time point of development of the amoeba. A bioinformatic prediction of expressed box H/ACA snoRNA candidates however surprisingly failed. We therefore have isolated box H/ACA snoRNP by immunoprecipitation (IP) using an antibody against the catalytic dyskerin. The IPed RNA was subjected to deep sequencing, result of which are currently under analysis.
Using homology-based models for the D. discoideum rRNA secondary structures, we localize many modified nucleotides in the vicinity of the ribosomal A, P and E sites. Our data from D. discoideum represents the first evidence for ribosome heterogeneity in the Amoebozoa supergroup, allowing to suggest that it is a common feature of all eukaryotes (2).

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(2) Diesend J, Birkedal U, Kjellin J, Zhang J, Jablonski KP, Söderbom F, Nielsen H, Hammann C. (2022) Fractional 2'-O-methylation in the ribosomal RNA of Dictyostelium discoideum supports ribosome heterogeneity in Amoebozoa. Sci Rep. 12:1952
34. Dictyostelium PRP4K is required for macropinocytosis and multicellular development

33: Dictyostelium PRP4K is required for macropinocytosis and multicellular development

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Splicing kinases are important regulators of spliceosome assembly and pre-mRNA splicing. Among the splicing kinases, pre-mRNA processing factor 4 kinase (PRP4K) has emerged as a regulator of diverse cellular pathways, including spindle assembly checkpoint, anoikis, Hippo signalling, and epithelial-mesenchymal transition in mammalian cells. Owing to its regulation of these pathways, PRP4K has been described as a tumour suppressor. However, PRP4K is an essential kinase in several common genetic model systems including flies, worms, and mice making it difficult to study loss-of-function phenotypes in these metazoan species. PRP4K is also conserved in single-celled eukaryotic species, including Dictyostelium discoideum, whose genome encodes a PRP4K ortholog, Prpf4B (DDB0216281). Using Dictyostelium as the model, we were able to generate a viable knockout of prpf4b using CRISPR/Cas9-mediated gene targeting. The prpf4b- mutant amoebae cannot grow in liquid culture, but growth can be rescued through the addition of heat-killed bacteria. We show that impaired macropinocytosis is likely responsible for this growth phenotype. In addition, when prpf4b-null cells are grown in flasks, large multinucleated aggregates form due to impaired cytokinesis, mirroring previous studies in mammalian cells depleted for PRP4K. During multicellular development, prpf4b-null amoebae display defects in streaming, mound formation and fruiting body formation. In conclusion, the Dictyostelium model facilitated the first study of PRP4K functions in the context of a complete knockout, indicating prpf4b has functions in both the growth and developmental phases of the life cycle.

34. Evolution of microRNAs in Amoebozoa

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MicroRNAs (miRNAs) are important regulators of gene expression in both plants and animals. They are thought to have evolved convergently in these lineages and hypothesized to have played a role in the evolution of multicellularity. In line with this hypothesis, miRNAs have so far only been described in a handful of unicellular eukaryotes. In this study we investigate the presence and evolution of miRNAs in Amoebazoa, focusing on species belonging to Acanthamoeba, Physarum, and dictyostelid taxonomic groups, representing true unicellular species as well as species where cells go through aggregative multicellularity. Through small RNA sequencing we identified miRNAs which adhere to both the stringent plant and animal miRNA criteria in nearly all examined amoebae, greatly expanding the total number of protists featuring miRNAs. We found conserved miRNAs between closely related species, but the majority of species feature only unique miRNAs. The number and expression of miRNAs seems to vary greatly between related species, and even the mode of targeting appears to differ. This is also reflected in the variable number of genes important for miRNA function, i.e. genes for Dicers and Argonautes. In one of the amoebae that features aggregative multicellularity, Dictyostelium discoideum, we observed no effect on the multicellular development when miRNA expression was abolished. Together our results suggest that miRNAs appear to have evolved independently in different amoebazoan lineages where they fulfil specific roles, but they do not seem to play a role in the evolution of multicellularity

35. Abundantly expressed class of noncoding RNAs conserved through the multicellular evolution of dictyostelid social amoebas

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Recent years' discovery of numerous non-coding (nc)RNAs has dramatically changed the view of how organisms control their biological processes. We previously identified an abundant and highly expressed class of ncRNA, 42 – 65 nt long, involved in multicellular development of the social amoeba Dictyostelium discoideum. In order to understand if Class I RNAs are unique to D. discoideum or also present in other organisms, we searched for Class I RNA genes in genomes from species representing each major group of dictyostelid social amoeba and numerous Class I RNA genes were identified in all of these species. In contrast, genomes from strictly unicellular Amoebozoa showed no evidence of this class of RNA. Analysis of Class I RNAs from the different social amoeba species revealed several conserved features. They harbor a short stem-structure, connecting the 5′ and 3′ ends, and a conserved sequence element. In addition, the genes are preceded by a putative promoter sequence. Our results show that Class I RNA is an ancient class of ncRNAs, likely to have been present in the last common ancestor of Dictyostelia dating back at least 600 million years and we hypothesize that Class I RNAs are involved in evolution of multicellularity in Dictyostelia. Presently we are analyzing mass-spec and RNA-seq data from wt cells and a strain depleted of one Class I RNA gene that we know affect early multicellular development. We expect this study to give insights into the workings of Class I RNA during multicellular development.

Ref: Kjellin J et al (2021) Abundantly expressed class of noncoding RNAs conserved through the multicellular evolution of dictyostelid social amoebas. Genome Res. 31: 436-447

36. Centrosome positioning in migrating Dictyostelium cells

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Directional cell migration and establishment of polarity play an important role in development, wound healing, and host cell defense. While actin polymerization provides the driving force at the cell front, the microtubule network assumes a regulatory function in coordinating front protrusion and rear retraction. By using Dictyostelium discoideum cells as a model for amoeboid movement in different 2D and 3D environments, the position of the centrosome relative to the nucleus was analyzed by live-cell microscopy. Our results show that the centrosome is preferentially located rearward of the nucleus under all conditions tested for directed migration, while the nucleus is oriented toward the expanding front. When cells are hindered from straight movement by obstacles, the centrosome is displaced temporarily from its rearward location to the side of the nucleus but is reoriented within seconds. This relocalization is supported by the presence of intact microtubules and their contact to the cortex. The data suggest that the centrosome is responsible for coordinating microtubules with respect to the nucleus. In summary, we have analyzed the orientation of the centrosome during different modes of migration in an amoeboid model and present evidence that basic principles of centrosome positioning and movement are conserved between Dictyostelium and human leukocytes.

37. Nuclear envelope dynamics during semi-closed mitosis

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Dictyostelium cells undergo a semi-closed mitosis, in which the nuclear envelope (NE) remains intact but free diffusion between cytoplasm and the nucleus takes place initiated by centrosome insertion along with nuclear pore complex disassembly at the onset of mitosis. During interphase the centrosome is attached to the cytosolic face of the nucleus. At the onset of mitosis, the corona dissociates, and the core structure is inserted into the emerging fenestra in the NE. Besides we studied the behavior of several Dictyostelium nuclear pore complex (NPC) components by immunofluorescence microscopy and live cell imaging during interphase and mitosis. We could show that some nucleoporins (NUP62, NUP93) dissociated from the NPC at the onset of mitosis while others remained (NUP210) leading to a partial open NE.

Further fenestration of the NE takes place around the central spindle during karyokinesis. At the end of mitosis, the centrosome relocates to the cytoplasmic side of the nucleus and, in order to allow closure of the fenestrae, the central spindle needs to be disassembled. We have found that the AAA-ATPase DdSpastin is capable of microtubule-binding and severing and is likely to be involved in this process. Protein-protein-interaction of DdSpastin with the HeH/LEM-family protein Src1 as well as co-localization with the endosomal sorting complex required for transport (ESCRT) components suggesting that NE membrane remodeling at the end of mitosis is conserved among eukaryotes. Moreover, an enrichment of NUP210 and NUP93 at these sites suggests an additional function of NPC proteins in closure of the NE.

38. Mechanistic insight into suppression of polyglutamine aggregation by Srcp

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Huntington's disease is a neurodegenerative disease caused by the expansion and aggregation of the polyglutamine tract in the huntingtin protein. One potential therapeutic approach to treat Huntington's disease is to develop strategies that reduce mutant huntingtin (mHTT) aggregation. We and others have found that the model organism Dictyostelium discoideum naturally encodes proteins with long polyglutamine tracts but is resistant to aggregation of polyglutamine-expanded proteins. Work from our laboratory has identified serine-rich chaperone protein 1 (Srcp1) as a protein that is both necessary for Dictyostelium's resistance to mHTT aggregation and sufficient to impart resistance to other organisms. Despite its important function, the mechanism that Srcp1 utilizes to suppress mHTT aggregation is unknown. Here we provide evidence that Srcp1 utilizes an amyloid-like domain to suppress mHtt aggregation. Using in vitro mHtt aggregation assays we demonstrate that a synthetic peptide of the amyloid-like domain suppresses mHTT aggregation by inhibiting secondary nucleation. Interestingly, this peptide inhibits mHTT aggregation in a manner that is dependent upon the regions of mHTT that flank the polyglutamine tract and has no effect on the aggregation of pure polyglutamine tract. To better understand structural aspects that mediate Srcp1 function we have generated computational models of Srcp1. These models predict that a region in Srcp1's C-terminus forms a β-hairpin, consistent with an amyloid-like fold. We next used these models to guide our design of synthetic Srcp1 peptides to identify a peptide that maximally inhibits mHTT aggregation. Our results identified the minimal Srcp1 sequence necessary to inhibit mHTT aggregation and determined that a tandem repeat of this sequence was more efficient at suppressing mHTT aggregation. We next wanted to identify the minimal sequence that is sufficient to inhibit mHTT aggregation in human cells. Similar to our in vitro data, our results identified a twenty amino acid sequence of Srcp1 that is sufficient to suppress mHTT aggregation in human cells. Finally, to validate that this effect was not restricted to mHTT we tested our peptides on polyglutamine-expanded ataxin-3 (ATXN3) and found that it was sufficient to suppress ATXN3 aggregation. Together our data are consistent with Srcp1 utilizing an amyloid-like domain to suppress aggregation of polyglutamine-expanded proteins and provides insight into the molecular details of how Srcp1 suppresses polyglutamine aggregation.

39. Presence and function of bacterial Rhs toxins in social amoebas

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Bacteria live in complex environments where they have to compete with other microorganisms for resources and protect themselves from predators. To overcome this, many bacteria produce toxins which can either be secreted or delivered to nearby cells in a contact-dependent matter. However, the role of bacterial toxins extends beyond the use as weapons against antagonists. They can also affect the life-style of the toxin producing bacteria by e.g., regulating growth rate or affecting multicellular behavior such as biofilm formation. One such example is found in Myxococcus xanthus, which is known for its ability to aggregate together and form fruiting bodies upon starvation. The fruiting bodies normally contains sporulated bacteria. However, if the Rearrangement hotspot (Rhs) toxin is disrupted, a large reduction in the number of spores is detected.
The multicellular development of M. xanthus resembles the development of dictyostelid social amoebas. Almost all social amoebas differentiate into at least two cell types during the development, stalk cells and spore cells. However, in one subgroup, Acytostelia, this ability has been lost and instead all cells form spores which are supported by an acellular stalk.
In this study we investigate the presence of Rhs in Dictyostelia and attempt to understand its function. We have identified and validated the presence of an Rhs homologue in A. subglobosum and by searching for Rhs associated domains in 22 dictyostelid genome assemblies we could identify Rhs presence in one additional acytostelid. Furthermore, we have sequenced the genome of Acytostelium digitatum with Oxford Nanopore technology and achieved a near chromosome-level assembly. Our ongoing experimental and comparative genomics work aims to elucidate the presence and role of Rhs toxins in the Acytostelia subgroup.

40. Identification of a new actin polymerization function of the WRC independent of the VCA domain, but dependent on its polyproline domains

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Cell migration requires the constant modification of cellular shape by reorganization of the actin cytoskeleton. Fine tuning of this process is critical to ensure new actin filaments are formed only at specific times and at defined regions of cell. The Scar/WAVE regulatory complex (WRC) is the main catalyst of pseudopods and lamellipodia formation. It is a stable pentameric complex highly conserved through eukaryotic evolution and composed of Scar/WAVE, Abi, Nap1/NCKAP1, Pir121/CYFIP and HSPC300/Brk1. Its actin nucleation activity has been attributed to its ability to combine monomeric actin and Arp2/3 complex through Scar/WAVE's VCA domain, while other regions of the complex are typically thought to mediate spatial-temporal regulation and have no direct role in actin polymerization.
Here we show that the WRC with its VCA domain deleted can still induce the formation of morphologically normal pseudopods and restore cell motility in Dictyostelium discoideum Scar KO cells. These results were further confirmed using WASP KO cells to avoid WASP ability to replace Scar at the leading edge of protrusions. We also expressed Scar/WAVE with VCA and polyproline domains both deleted. However, this construct was able to rescue pseudopod formation only if WASP was available. Similar results are seen in B16-F1 mouse melanoma cells: the WRC with its VCA domain deleted is able to catalyse the formation of actin protrusions, while deletion of both Abi and WAVE proline-rich domains completely abolishes the complex's function. Thus we conclude that proline-rich domains play a central role in actin nucleation.
Our data demonstrate a new actin nucleation mechanism of the WRC that is independent of its VCA domain. We also show that proline-rich domains are more fundamental than has been thought. Together, these findings suggest a new mechanism for WRC action.

41. Predicting novel migration behaviours

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Directed cell motility plays a crucial role at some stage for most forms of life. Single celled organisms rely on finely controlled motion to hunt or migrate toward optimum environmental conditions. Multicellular organisms require a continual system of simultaneous - and precise - cellular migration, from conception until death, simply in order to develop and survive. These migrations are most often modulated via diffusible extracellular signals, causing the affected cell to move towards or - rarely - away from the source of the signal; this is chemotaxis. The primary aim of this thesis is to come to a fundamental understanding of the relationship between chemotactic signals and the elicited cellular response, from basic singular signals to complex multi-signal systems, and use this knowledge to predict novel migration behaviours. This is achieved using a feedback loop between an iterative process of mathematical modelling, to predict chemotactic responses, and live cell imaging, to validate and improve upon the model. Assays are performed using Insall chambers, a direct visualisation chemotaxis chamber, and the motile amoeba Dictyostelium discoideum. A diverse range of extracellular signalling conditions are constructed via the varying chemotactic properties of different derivatives of cAMP, a signalling molecule to which D. discoideum are highly chemotactic. Using mathematics as an interface between chemical concentration and subsequent cell-surface receptor activation, many complex migration behaviours were predicted and experimentally verified. Of particular interest are: a robust system by which chemorepulsion - the mechanics of which have been elusive - can be mediated, conditions that can give rise to an inversion of chemotactic directionality in the same gradient, and proof that two signals that cause chemoattraction in isolation can be combined to induce chemorepulsion.

42. How phagocytes acquired the capability of hunting and removing pathogens: lessons learned from chemotaxis and phagocytosis of Dictyostelium discoideum

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How phagocytes find invading microorganisms and eliminate pathogenic ones from human bodies is a fundamental question in the study of infectious diseases. About 2.5 billion years ago, eukaryotic unicellular organisms --protozoans-- appeared and started to interact with various bacteria. Less than 1 billion years ago, multicellular animals --metazoans-- appeared and acquired the ability to distinguish self from non-self and to remove harmful organisms from their bodies. Since then, animals have developed innate immunity in which specialized white-blood cells -phagocytes- patrol the body to kill pathogenic bacteria. The social amoebae Dictyostelium discoideum are prototypical phagocytes that chase various bacteria via chemotaxis and consume them as food via phagocytosis. Recently, we discovered that D. discoideum amoeba use a chemoattractant GPCR fAR1 to detect folic acid released from bacteria for both chemotaxis to catch bacteria and phagocytosis to ingest them. This finding suggest to us that a chemoattractant GPCR-mediated signaling network controls reorganization of the actin cytoskeleton for both chemotaxis and phagocytosis, which represents a paradigm-shifting new concept in immunology. Thus, investigation of chemotaxis and (or) phagocytosis in D. discoideum will continually shed light on the molecular mechanisms controlling migration of immune cells and as well as phagocytosis by immune cells to eliminate bacterial pathogens from human body.

43. Observing cellular slime mold aggregation and signaling in transparent soil microcosms

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In nature, cellular slime molds grow, aggregate, and develop in highly complex, heterogeneous microenvironments in soil and dung. This raises two fundamental questions: first, how are population-scale patterns during development shaped by properties of the local microenvironments where cells interact? Second, what cell-level features allow aggregation to be robust to the environmental heterogeneity present in natural microenvironments? To address these questions, we developed a novel system to observe and quantify growth, aggregation behavior, and cAMP signaling in Dictyostelium discoideum in transparent soil microcosms. We demonstrate the utility of these microcosms to observe cell and population-level behaviors in naturalistic environments. The microcosms also allow for experimental manipulation of specific features of the system, like the particle size distribution and cell density at aggregation. We will use this system to learn generalizable rules about (1) how features of the environment, together with cell-level properties, shape multicellular outcomes and (2) what allows behaviors to be robust to environmental heterogeneity.

44. dictyBase (DCR) updates

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GWDI strain, advanced search and order workflow
In this update of DCR (dictyBase), we have integrated the GWDI mutants in our strain catalogue and provide a user intuitive interface for searching the entries. In addition, we have installed a refined Dicty Stock Center (DSC) workflow to order the strains from the catalogue. The GWDI strains are available through our strain catalogue, and they can be displayed by selecting the "GWDI" entry in the dropdown menu of catalogue. Like "GWDI", every menu entry represents a dataset, and it can be further refined by a specific any property such as descriptor, summary, or availability. Every property is also a searchable field, and the available field pops up as soon as the user clicks the search box. In the search, user may select to query any combination of available fields. Every combination of fields and query text is shown in a separate chip style display element inside the search box. Each of the chip elements is removable by user click and once it is removed the search query and catalogue display is adjusted accordingly. The Plasmid catalogue works the same way. Any order from the catalogues will send an email confirmation to both the user and the DSC with a customized PDF attachment containing order details. Users can now also directly download a PDF receipt after submitting their order on the DSC website.

Curation web interface
As a first step for curation of the new database we implemented a new web interface that lets us import data we recently added into Excel tables and allows curators to add new data one by one. The data can be viewed in different ways and certain columns may be hidden when used for display (such as IDs). We will demonstrate adding phenotype data. The import into the new database will be easily accomplished via an API.

45. Dictyostelium and acanthamoeba present different motility response to hypoxia and oxygen gradients.

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Both the social Dictyostelium and the solitary acanthamoeba are obligatory aerobic amoeba that live in the soil and feed on other microorganisms. State of low oxygen occurs frequently in soil due to waterlogging or respiration by large amount of other microorganisms. We have previously shown that when oxygen level is less than 2%, Dictyostelium cells will increase their motility and move up oxygen gradients, a phenomenon called aerotaxis. As a result, confining a micro-colony of Dictyostelium cells triggers a quick outward movement out of the self-generated central hypoxia area and form an dense ring of cells on the outside border. By contrast, confining a micro-colony of acanthamoeba will result in a very rapid but disorganized outward movement. To model and explain these behaviors, we used microfluidic device and hypoxic chambers and explored the differential response to oxygen between both amoeba. Acanthamoeba moves with higher speed and persistence that dictyostelium and is areatoactic up to 5-10% oxygen. It is possible that comparatively to acanthamoeba, the social Dictyostelium cell were selected during evolution to tolerate hypoxic condition due to neighboring cells, resulting into a narrower range of aerotactic response. Furthermore, our microfluidic experiments allowed to exclude the possibility that the aerotactic response is due to a secondary H202 or NO gradient.

46: The Dictyostelium Zinc Finger Protein TtpA Regulates the Co-ordinated Stability of a Group of mRNAs, through a Common 3' UTR sequence

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Post-transcriptional processes mediated by mRNA binding proteins represent important control points in gene expression. In eukaryotes, mRNAs containing specific AU-rich motifs are regulated by binding of tristetraprolin (TTP) family tandem zinc finger proteins, which promote mRNA deadenylation and decay; in contrast to the multi-members expressed in humans and most other eukaryotes, the Dictyostelium discoideum genome encodes only single TTP family member, TtpA. Evaluation of ttpA null-mutants identified six transcripts that were consistently upregulated compared to WT during growth and early development. The 3'-untranslated regions (3'-UTRs) of all six "TtpA-target" mRNAs contained multiple, overlapping UUAUUUAUU motifs, which bound TtpA in vitro; one 3'-UTR conferred TtpA post-transcriptional stability regulation to a heterologous mRNA, and where regulation was abrogated by mutations in the core TTP-binding motifs and in ttpA null-mutants. In Dictyostelium, TtpA may control expression levels of functionally related gene sets, where the RNA binding protein TtpA post-transcriptionally co-regulates mRNA stability

47. From pseudopod to chemotaxis

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Twenty years ago I thought that cells in buffer extend pseudopods randomly, while in a chemotactic gradient the direction of pseudopod extension is regulated by a complex signal transduction network. However, ten years of pseudopod analysis in wild type cells and dozens of mutants show the opposite. Pseudopod formation in buffer is far from random and is highly regulated in time and space. Similar observations were made for Dictyostelium, neutrophils, stem cells and the fungus Bdxx. Time: Cells without an extending pseudopod have a high probability to start one, which immediately inhibits the start of a second pseudopod. This inhibition depends on the parallel F-actin/myosin/formin cortex; mutants lacking one of these components simultaneously extend many pseudopods and move in a chaotic manner. Space: In buffer pseudopods are preferentially made in the front half of the cell, because cells are polarized, and in the front pseudopods are extended alternatingly to the left and right. Polarization is mediated by cGMP and depends on parallel F-actin/myosin, which inhibits the start of a new pseudopod in the rear of the cell. This polarization functions as a long-term memory and is stable for about 2 minutes, remembering and averaging the direction of the last ~6 pseudopods. The pseudopod in the front start at the place where the pre-previous pseudopod has stopped, which is due to the stimulatory remains of that pseudopod, notably the phosphorylation state of SCAR. This functions as a short-term memory of ~20 seconds, remembering only the last pseudopod. The consequence of short- and long-term memory is that cells move in a persistent manner in the direction of the past ~6 pseudopods.

The main regulators of pseudopod induction in buffer are an excitable field of Ras/SCAR/F-actin. Pseudopods start at a place of a Ras-GTP patch that activates Rac, Scar and Arp2/3 leading to branched F-actin in the emerging pseudopod. Due to the excitable medium, Ras patches preferentially appear at places with elevated levels of Scar or branched F-actin, thus at the place with remains of a previous pseudopod. The consequence of temporal and spatial control of pseudopod formation in buffer is that cells preferentially extend only one pseudopod that is formed at places of Ras patches that occur in the front where a previous pseudopod was made. During chemotaxis, cAMP induces local Ras activation that combines with the internal Ras activation that would occur in buffer, leading to a small shift of the maximal Ras activity to the right or left, and thereby to a small bias of pseudopod extension in the direction of cAMP. This new direction is remembered and the next pseudopod would emerges at that position but again with a small bias in the direction of the gradient. Calculations show that the refined temporal and spatial regulation of pseudopods in buffer leads to an at least ten-fold increase of the sensitivity of the cells to respond to shallow cAMP gradients. Thus, the high efficiency of cAMP chemotaxis is fully based on the intricate temporal and spatial regulation of pseudopod formation in buffer.

48. The bubble wand model for micropinocytosis

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In macropinocytosis, cells engulf droplets of medium into micron-sized vesicles, then digest the contents for further utilization. Amoebae and cancer cells use macropinocytosis for feeding and immune cells to take up antigens; whereas viruses and bacteria use it as a back door to infect cells. Conversely, macropinocytosis is an entry route for drugs, vaccines and therapeutic mRNA. Despite this clear medical importance, and its discovery nearly 100 years ago, only recently has macropinocytosis started to attract major attention. Fundamental questions therefore remain, such as how are macropinocytic cups formed; what supports them; what makes them close; and how do they close?

Macropinocytosis is conserved across animals and amoebae. It is driven by the actin cytoskeleton and PIP3 plays a crucial role: in Dictyostelium it forms domains, first discovered more than 20 years ago, around which macropinocytic cups form. Macropinocytosis can be observed by lattice light-sheet microscopy, which unlike most other forms of microscopy, allows cups to be followed in 3D over their entire life span, from origin to closure. We collected over a thousand movies made this way, using around 20 reporters and 15 strains, and analyzed them with custom software.

We find that macropinocytic cups can expand from small origins and close in two different ways: either by constriction of their rim, caused by inwardly-directed actin polymerization; or stretching and fission of their base. A wild-type NC4 derivative performs macropinocytosis in a similar way to axenic cells, showing that these phenomena are general. Cups are supported by a continuous F-actin scaffold to which the membrane is firmly bound. PIP3 domains extend to the lip of cups, where Scar/WAVE and Arp2/3 are recruited. This recruitment produces a hollow ring of actin polymerization around PIP3 domains, which we propose is the means by which cups are templated in the plasma membrane.

PIP3 domains were ‘dissected’ out computationally by a double-segmentation procedure and their surface area and other geometric properties measured through their life. This shows that in early life, cups expand and remain relatively shallow, with an outward wave of actin polymerization capturing membrane. Eventually the wave slows and stalls, and the cup deepens, then closes.

Based on our observations we propose three principles that help explain macropinocytosis:
- PIP3 domains create a hollow ring of actin polymerization around themselves
- cups are supported by an F-actin matrix to which the membrane of the PIP3 domains is firmly linked
- cups close when the PIP3 domains cease expanding (‘stalled wave’)

These principles can be embodied in a simple physical model – the ‘bubble wand model’ - that recreates many of the phenomena we observe.

Poster Abstracts

1. A systematic analysis of the G-protein coupled receptors of the dictyostelids and their implications on the evolution of multicellularity

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G-protein coupled receptors (GPCRs) are a conserved family of seven-transmembrane receptors that transduce signals via small trimeric G proteins and constitute the largest family of membrane proteins in eukaryotes. GPCRs perform many important roles in environmental sensing and general cell-cell communication. They are divided into six classes: class 1 rhodopsin-like, class 2 secretin-like, class 3 metabotropic glutamate receptors, class 4 fungal pheromone mating factor receptors, class 5 frizzled/smoothened GPCRs and class 6 dictyostelid cAMP receptors. We have analysed the phylogeny-wide conservation and change in the GPCRs across the dictyostelids including some amoebozoan outgroups and found deep evolutionary conservation of most GPCR classes. The cAMP receptor-like class 6 was thought to be exclusively present in the dictyostelids and the class 4 pheromone receptor GPCRs restricted to the fungi. In this study, we demonstrate for the first time the existence of rhodopsin-like class 1 GPCRs in the dictyostelids. The class 1 GPCRs have roles in sensing hormones, neurotransmitters, small molecules and light in other organisms but their roles have not yet been analysed in *Dictyostelium*. Analysis of their expression patterns shows that some of them are upregulated during multicellular development and could play roles there. Consistent with previous studies (Prabhu and Eichinger 2006, Heidel et al. 2011) we found class 2, class 3, class 5 and class 6 GPCRs in all groups dictyostelids, but no class 4 fungal pheromone receptors. Many of the class 6 GPCRs represent well-studied cAMP receptors perform essential functions at the multicellular stage. Here, we show that this receptor family is not restricted to the dictyostelids as previously thought, but also present in *Physarum polycephalum, Protostelium aurantium* var. fungivorum and *Acanthamoeba castellanii* suggestive of an earlier origin of this receptor class. Our analysis points out where GPCRs have been conserved throughout the evolution of dictyostelids and highlights where group-specific amplification and losses occurred. We have also analysed their expression patterns and found that most GPCRs are expressed during multicellular development with most showing some cell-type specificity. This suggests that the expansion of GPCR types at the root of the dictyostelids could have been driving the evolution of multicellularity.

2. National BioResource Project Nenkin (NBRP Nenkin): The Bioresouce Bank of Cellular Slime Molds in Japan

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National BioResource Project of cellular slime molds in Japan (NBRP Nenkin) serves as a bioresource center since 2007. Many Japanese scientists have been studying various topics in broad research fields using Dictyostelium discoideum and other slime molds, therefore we have reserve and serve many individual cellular slime mould cells and related plasmids. These activities have produced many important bioresources. Our aim is to collect these bioresources and to distribute them to the Dicty research community all over the world to accelerate research using especially Dictyostelium discoideum cells. So far, we have collected and stored around 1,400 strains and around 650 plasmids which are mainly from Japanese laboratories.
From 2022, we, NBRP, renewed and university of Tsukuba take over the project from RIKEN. In these several year, all-in-one vectors for CRISPR/Cas9-mediated gene modification including knockouts, inducible knockouts, knockdowns, knock-ins, point mutations and deletions, were deposited (Sekine et al., Sci. Rep., 8, 8471, 2018; Yamashita et al., Front. Cell Dev. Biol., 9, 721630, 2021; Asano et al., Sci. Rep., 11, 11163, 2021). These plasmids have been ordered from all over the world. We also collected a highly bright and sensitive cAMP live-reporter, Flamindo2, from Drs. Yusuke Morimoto and Masahiro Ueda (Hashimura et al., Communication Biology, 2, pp1, 2019). Flamindo2 can detect changes in the cytosolic cAMP concentration from ~0.3 -12 mM in the early aggregation stage and mound stage. We hope they will help your research. Furthermore, the stocked strains include field-isolates which are currently contributing to discovery of pharmacological lead compounds for drug development. All resources in NBRP are available to any person through the website (http://nenkin.nbrp.jp). Please visit the website of NBRP which may be useful for your work. Furthermore, we accept your deposits of bioresources of your studies from around the world. If you have submissions, please contact us (nbrpnenkin@shigen.info, or kuwayama.hidekazu.fu@u.tsukuba.ac.jp). We would be grateful for your contribution to this project and comments are welcomed.

3. Extracellular adenosing deamination primes tip organiser development in *Dictyostelium*

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It is a long-held view that ammonia generated by proteolytic activity drives tip organizer development in *Dictyostelium* and our results demonstrate that extracellular adenosine deamination also contributes to tip development. Adenosine deaminases (ADA) convert adenosine to inosine and generate ammonia as a by-product and adenosine deaminase related growth factor (ADGF), belongs to a family of growth factors containing an adenosine deaminase domain. The *Dictyostelium adgf-* mutants are arrested as rotating mounds, blocked in tip development and wild type cells treated with an extracellular ADA inhibitor, deoxycoformycin, phenocopied the mound arrest defect. Adenosine levels in *adgf-* cells are significantly high, and adding ADA enzyme directly on the mutant mounds restored tip formation, indicating that the inhibition of ADA activity is responsible for the mound arrest phenotype. Since cAMP signaling is known to exert a significant influence in tip development, we quantified the expression of adenylyl cyclase A (*acaA*) and cAMP levels in *adgf-* mutants and found their levels to be low. The mound arrest could be rescued by adding 8-Br-cAMP, a cell permeable cAMP analog. The cAMP wave propagation as visualized by dark field optics shows circular wave pattern in *adgf-* mutant as compared to the spiral wave pattern in wild type AX4. Addition of cyclic di-GMP known to activate adenylate cyclase also initiated tip formation. Interchanging the conditioned medium (culture supernatant) and reconstitution with wild type AX4 cells restored tip formation of *adgf-* cells suggesting that *adgf-* phenotype could be due to lack of production of a secreted signal. To confirm if adenosine deamination alone is responsible for rescuing the mound defect, AX4 and *adgf-* cells were developed in a compartmentalized but sealed petri dish and AX4 on one side of the dish rescued the phenotype suggesting that volatile ammonia generated from the wild type cells is rescuing the defect. To understand the pathway of how ADA controls tip formation, the enzyme ADA was directly added to mutants with similar developmental defect as *adgf-* and we identified the histidine kinase *dhkD* as a potential downstream target. Further, the prestalk gene expression is upregulated and prespore expression is downregulated in *adgf-* compared to AX4 which suggests that *adgf-* is involved in cell type patterning. Our work further suggests that the successive transformation events of cAMP to adenosine and adenosine to ammonia are sequentially integrated in the consecutive steps of *Dictyostelium* development.

4. Deciphering primary events in the evolution of phototrophic endosymbionts in a Synechocystis-Dictyostelium model

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Eukaryotes harbour organelles such as mitochondria and chloroplasts that originate from endosymbiosis of a proteobacterium and a cyanobacterium, respectively. Although these events have been the foundation of eukaryotic life forms, there are several open questions regarding these initial processes of organelle formation.

The objective of the project is to identify conditions and molecular interactions that have favoured the establishment of a mutualistic partnership between a photoautotrophic prokaryote and a heterotrophic phagocytic amoeba. The cyanobacterium Synechocystis sp. PCC 6803 and the phagocyte Dictyostelium discoideum are both model organisms, each one offering the full array of molecular tools, which are necessary to tackle fundamental questions in the coevolution of host and endosymbiont. Moreover, they offer the possibility to study a bipartite interaction at the scale of entire populations down to the single cell level.

We initially found that Synechocystis was ingested by D. discoiduem, but not utilized as a preferred food source by the amoeba when compared to γ-proteobacteria. Following ingestion, the cyanobacteria were processed in phagolysosome of Dictyostelium. Processing resulted in two different major scenarios: The bacteria were either rapidly expelled from the phagocyte via exocytosis or resided in phagolysosomes. Cellular retention of the bacteria resulted in rapid loss of motility and finally lysis of the amoeba host cell. Analyses of cellular extracts of the cyanobacteria revealed no amoebicidal metabolic products suggesting an interactive biochemical process between the two partners. We next varied abiotic conditions, such as light and CO2. Loss of motility and cellular lysis were drastically accelerated with phagocytically active amoebae in the light. We hypothesize this is due to the photosynthetic production of reactive oxygen species (ROS) of the cyanobacterium, making it plausible that detoxification of ROS in early eukaryotes were among the most significant challenges to overcome.

5. Autophagy of dictyostelid stalk cells feeds the spores

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Autophagy assists survival of starving cells by partial self-digestion, while dormancy as cysts or spores enables long-term survival. Starving Dictyostelium amoebas construct fruiting bodies with spores and stalk cells, with some groups having additionally retained ancestral encystation as single cells. While autophagy mostly occurs in the somatic stalk cells, autophagy gene knock-outs in D. discoideum formed no spores and overproduced stalk-like cells. Strikingly, loss of autophagy specifically prevented cAMP induction of prespore gene expression. To investigate whether this effect is conserved across Dictyostelia and whether autophagy also prohibits encystation, we deleted autophagy genes atg5 and atg7 in Polyspondylium pallidum, which forms both fruiting bodies and cysts. Loss of atg5 or atg7 reduced, but did not prevent, encystation and cysts were less viable. However, sporulation and cAMP induction of prespore gene expression were completely lost. Spores are more resilient than cysts and have thicker more structured walls. We tested whether spore dependence on autophagy signifies that nutrient flux from stalk cells nurtures the spores. Sporulation requires secreted cAMP acting on receptors and intracellular cAMP acting on PKA. We compared viability of spores developed in fruiting bodies with spores induced from single cells after stimulation with cAMP and 8Br-cAMP, a PKA agonist. The walled spores formed in isolation were small and did not germinate after detergent treatment like multicellularly developed spores, indicating wall deficiencies. In context with species ecology, this suggests that stalk cells not only lift but also feed the spores, highlighting autophagy as an ultimate cause for soma evolution.

6. Why doing it again? - How the CryoEM structure of native SCAR/WAVE complex can further elucidate its function

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The actin cytoskeleton fulfils multiple functions within eukaryotic cells including vesicle transport, cell division and migration. One of its major regulators is the SCAR/WAVE complex (short: WRC for – wave regulatory complex) which promotes actin nucleation and so the assembly of branched actin filaments. Those filaments are crucial for the formation of pseudopods and lamellipods, cellular protrusions which facilitate cell movement. Cells lacking the WRC are unable to form these protrusions, making it a key regulator of cell migration.

How does the complex promote actin polymerization? The hetero-pentameric complex consists of two subunits. The larger dimer is formed by PirA and Nap1 while SCAR/WAVE, AbiA and HSPC300 form the smaller trimer. The activation of the complex is supposed to be mediated by the binding of GTP bound rac1 via the PirA subunit. Conformational changes of the complex result in the release of the VCA domain of SCAR/WAVE which recruits the Arp2/3 and leads to actin polymerization.
However, current research suggests that this is not the only route to promote actin polymerization in pseudopodia and lamellipodia. Recent data shows that the poly-proline domains present in SCAR/WAVE and AbiA are sufficient to drive actin polymerization even in the absence of the VCA of SCAR/WAVE.

These novel findings created the necessity to revisit the existing crystal structure of the WRC. Based on the assumption that the poly-proline domains only function as passive linkers and to support the crystallization of the complex a so called mini-WRC was created. It lacks both, the poly-proline domains of SCAR/WAVE and AbiA and the VCA itself to keep the complex inactive. The assembly of the mini-WRC occurred in vitro in the absence of WRC specific chaperones.
We have optimized a new purification protocol for Dictyostelium cells that allows us to purify native WRC from motile cells. This gives us for the first time the possibility to look at non-truncated functional WRC-complex in high resolution using cryoEM. Our approach aims to identify the exact positions of the poly-proline domains as well as the VCA to get a clearer picture how the WRC promotes actin polymerization.

7. Identification and validation of substrates for phosphorylation by PKA in Dictyostelium discoideum

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PKA is one of the most important protein kinases regulating Dictyostelium multicellular development, but its phosphorylation substrates remain largely undiscovered. In this work, we adopted 2 phosphoproteomics screens to detect the phosphopeptides arisen from PKA phosphorylation in vivo and have identified 12 PKA phosphorylation targets: 4 metabolic enzymes and 8 signal transduction proteins. No gene regulatory/transcription factors were detected, which may be due to their low abundance in Dictyostelium cells. We validated four putative PKA targets in vitro by a thio-phosphorylation reaction, incubating synthetic peptides with a modified PKA (PKA-F413G or AS) and a bulky ATP analogue (6-benzyl-ATPγS). We complemented null mutants of two candidate PKA targets (UgpB and RasGefM) by transformation with wild-type and non-phosphorylatable forms of the target proteins and found that phosphorylation of the proteins was required for complementation and acted to protect the proteins from degradation.

Keywords: PKA, phosphorylation/thio-phosphorylation, protein stability, Dictyostelium.

8. Superresolution Expansion Microscopy in Dictyostelium Amoebae

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Expansion microscopy (ExM) is a superresolution technique for fixed specimens that improves resolution of a given microscopy system approximately four-fold. The gain in resolution in ExM is not achieved by improvement of the resolution of the microscope itself but by isotropic expansion of the sample. To achieve this, the sample is crosslinked to an expandable gel matrix that swells approximately four-fold by incubation in water. We have applied the method to Dictyostelium amoebae and discuss and show the pros and cons of different labeling techniques in combination with pre- and post-expansion staining protocols.

9. Phenotypic characterization of a TSPOON mutant in *Dictyostelium discoideum* with a perspective on multilamellar body production

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Dictyostelium discoideum produces multilamellar bodies (MLBs) that are composed of concentric lipid membranes when cultured on digestible bacteria. The role of MLBs remains elusive as well as the mechanisms behind their production. In the present ongoing study, we explored the potential role of the mutant strain tstD- in this process and in the cell physiology in general. This mutant lacks a functional gene coding for the protein TSPOON, a protein involved in vesicle trafficking. Various phenotypes have been analyzed so far. Osmoregulation and cytokinesis were not altered in tstD- compared to the parental cells. A predation assay on various bacterial lawns suggests that there is no significant difference in the ability of tstD- to prey upon the bacteria tested in comparison with Ax2, but the morphology of the phagocytosis plaques of tstD- was different on the strain Aeromonas salmonicida subsp. salmonicida HER1110 compared to Ax2 cells. The growth speed of tstD- was also assessed and it turns out that this mutant has an increased growth rate both in agitated liquid culture and on a bacterial lawn of Klebsiella aerogenes. Still on K. aerogenes, tstD- was unable to produce mature fruiting bodies. Both in Ax2 and tstD-, the MLBs were harboring the classic concentric membrane morphology with no significant difference in terms of size, density, intracellular location and form. The amount of MLBs secreted by tstD- and Ax2 was similar. This ongoing work on the characterization of tstD- highlighted new phenotypes for this mutant, including an altered multicellular development.

10. Optogenetic manipulation of molecular oscillation during development

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Biological oscillations are considered as periodic dynamics at various levels of cellular processes, such as the circadian rhythm, segmentation clock, and activation of neural stem cells. A GATA transcription factor, namely, GtaC, may control the developmental timing of Dictyostelium through nucleocytoplasmic shuttling in response to the spatiotemporal pattern of cAMP waves. During early development, cAMP waves gradually become frequent, whereas the frequency-dependent response of GtaC shuttling remains unclear. In this study, we visualised the spatiotemporal dynamics of endogenous GtaC by using mNeonGreen knock-in cell lines generated by CRISPR/Cas9. Moreover, we manipulated the frequency of intracellular cAMP levels using an optogenetic tool to determine whether GtaC exhibits “low-pass filter” properties for its proper transition to the multicellular stage.

11. Exploring the impact of *cln5* loss on gene expression, protein levels, and enzymatic activity during *Dictyostelium* growth and development

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The ceroid lipofuscinosis neuronal (CLN) proteins (CLN1-CLN8, CLN10-CLN14) are a family of proteins associated with Batten disease (neuronal ceroid lipofuscinosis) in humans. One of these proteins, CLN5, localizes to the lysosome and extracellularly, and is thought to function as either a glycoside hydrolase or depalmitoylase. Previous research showed that CLN5 participates in a variety of cellular processes including, but not limited to, lipid metabolism, biometal homeostasis, and autophagy. However, the precise function of CLN5 in the cell and the pathway(s) regulating its function are not well understood. Loss of the CLN5 homolog in Dictyostelium discoideum, cln5, causes several aberrant phenotypes including reduced cell proliferation, aberrant cytokinesis, delayed aggregation, reduced cell adhesion, and accelerated multicellular development following mound formation. In this study, we used comparative transcriptomics to identify differentially expressed genes underlying these phenotypes. During growth, genes associated with ubiquitination, deubiquitination, cell cycle progression, and proteasomal degradation were affected, while genes linked to protein and carbohydrate catabolic processes were affected in cells starved for 4 hours, which is the life cycle stage where cln5 is maximally expressed. We followed up this analysis by showing altered intracellular and extracellular amounts of autocrine proliferation repressor A and counting factor-associated protein D in cln5- cells during growth. In addition, we detected reduced amounts of cortexillin B in cln5- cells, but increased amounts of myosin heavy chain, which are both linked to cytokinesis. During starvation, loss of cln5 increased the extracellular amount of conditioned media factor (CmfA), which regulates cAMP signaling. Additionally, cln5- cells displayed increased amounts of discoidin, a protein involved in cell-substrate adhesion and cellular migration, inside and outside the cell. Previous work in other cellular models reported altered lysosomal enzyme activity due to the mutation or loss of CLN5. Here, we detected altered intracellular activities of various glycoside hydrolases (e.g., α-mannosidase, α-glucosidase, β-glucosidase, and N-acetylglucosaminidase), cathepsins (CtsB, CtsD), and cysteine proteinases (CprA, CprB, DDB0252831) during cln5- growth and starvation. Finally, consistent with the differential expression of genes associated with proteasomal degradation in cln5- cells, we also observed elevated amounts of one of the proteasomal subunits and reduced proteasomal activity during growth and starvation. Overall, this study reveals the impact of cln5-deficiency on gene expression and provides insight on the genes and proteins that play a role in regulating Cln5-dependent processes.

12. Cannabinoid research in D. discoideum

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Cannabis sativa has been used therapeutically for thousands of years, but derived phytocannabinoids such as cannabidiol (CBD) and cannabigerol (CBG) have only been recently introduced as pharmaceutical medicines. Both CBD and CBG have potential for the treatment of various diseases such as epilepsy, multiple sclerosis, and cancer, although the underlying mechanisms for these effects remain unclear. To address this, our research in D.discoideum has identified potential cellular mechanisms through which CBD and CBG treatment may provide some therapeutic effects. In D.discoideum, we have shown that CBD targets cell signalling regulated by the mitochondrial glycine cleavage system protein, GcvH1 which is orthologous to the human GcsH enzyme, that functions to metabolise glycine leading to the synthesis of methionine as part of folate one-carbon metabolism (FOCM). We have shown, for the first time, that CBD treatment reduces methionine levels in this model, and validated this effect using an ex vivo mouse hippocampal seizure model, linking this to functional activity in seizure control. In addition, using D. discoideum as a model, we have identified that both CBD and CBG treatment elevated the activity of the mechanistic target of rapamycin complex 1 (mTORC1) in wild-type cells. Surprisingly, this effect is reversed when levels of an upstream regulator of mTORC1, inositol polyphosphate multikinase (IMPK) are increased, such that CBD or CBG treatment decreases mTORC1 activity. This differential effect was then validated using primary human peripheral blood mononuclear cells (PBMCs) from healthy individuals and patients with multiple sclerosis, indicating a novel mechanism for the effects of CBD and CBG on mTORC1 regulation dependent on upstream IMPK signalling and disease state. Thus, our findings in D. discoideum identify two novel signalling pathways targeted by CBD and CBG, providing new insights into the therapeutic mechanisms of these cannabinoids in disease treatment.

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13. Phosporylation-dependent Protein-Protein-Interaction Studies of Centrosome Proteins

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Dictyostelium amoebae provide a useful model to study basic, centriole-independent processes in centrosome biology. The centrosome consists of a multi-layered core structure surrounded by a microtubule-nucleating corona. Our major long-term goal is to elucidate the molecular mechanisms involved in the duplication of this centrosome type. One key event taking place at the G2/M transition is the splitting of the layered core structure, whereby the central layer of the three major layers disappears. The central layer consists of three components, CP39, CP75 and CP91. All three are potential targets for regulation by mitotic kinases at the onset of mitosis. Based on results from other organisms and on own BioID protein- protein interaction studies, the three kinases Plk, CDK1 and Nek2 are candidate regulators. To identify putative target sites in CP39, CP75 and CP91 for the three kinases candidates, we used insilico tools like ELM and GPS-PBS. In each protein we focused on the regions known to be relevant for dissociation in mitotic centrosomes.
Now we want to mutate each possible target site and check for significant changes in protein-protein Interaction. For that, we generated a sixfold S/T to A and S/T to E point mutation in the centrosomal core layer protein CP39. This includes two possible phosphorylation sites for each kinase candidate. To evaluate changes in the interaction between CP39 with itself and another core-layer-protein CP75 we employ Yeast-Two-Hybrid assays. As a positive control we tested the CP39 wildtype against itself and CP75 in the Yeast-Two-Hybrid matchmaker gold system. In cases of positive interactions of the wildtype proteins, we plan to test the sixfold and all mutations alone in this assay. The results will be confirmed by BioID and pulldown assays.

14. The 'Tail' of Talin: Mechanical adaptation during the evolution of multicellular organisms

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The evolution of multicellular life dates back 700 million years ago. It has been recognized that, with the rise of multicellularity and the formation of tissues, cells became subject to increasingly complex mechanical forces. We thus hypothesize that multicellular organisms have evolved specific subcellular structures, for example at the plasma membrane and nuclear envelope, to cope with mechanical stresses. To test this hypothesis, we compare the subcellular mechanics of mammalian fibroblasts with that of the social amoeba Dictyostelium discoideum, which can exist in both unicellular and multicellular states. We focus our studies on the adhesion protein talin, a crucial integrin activator and force transducer in metazoans. D. discoideum expresses two talin proteins but lacks integrin receptors and instead binds Sib (Similar to Integrin Beta) proteins to establish a mechanical linkage between plasma membrane and cytoskeleton. Whether such talin-Sib linkages can indeed bear mechanical forces, and how such connections compare to talin-mediated cell adhesion complexes in mammals, remains unknown.
We therefore employ our previously developed, molecular tension sensors (1, 2) to investigate talin mechanics in mammalian cells, in D. discoideum, and in mammalian cells expressing chimeric talin proteins. Our preliminary results indicate that the talin rod domain of D. discoideum is capable of mediating mechanical linkages in fibroblast and can carry mechanical loads very similar to those observed across mammalian talin 1. However, the chimeric talin protein does not fully rescue the severe spreading phenotype of talin-deficient fibroblasts indicating that mammalian talin acquired additional functionalities to ensure cell adhesion maintenance in cells of higher animals. These experiments are currently complemented by the first molecular force measurement across talin A in D. discoideum.

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15. Copine C plays a role in substrate adhesion and streaming in Dictyostelium

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Copines make up a family of calcium-dependent membrane-binding proteins found in most eukaryotic organisms. Copine protein expression is upregulated in multiple human cancers and functional studies indicate the upregulation of copines promotes cell proliferation and migration. Humans have nine copines that are differentially expressed in various tissue types. The highly conserved nature of these proteins suggests a fundamental cellular function; however, a common mechanistic function for this family has not been identified. We are using the model organism Dictyostelium discoideum, which has six copines genes (cpnA-cpnF), to study these proteins. To investigate the role of copines in Dictyostelium, we generated a null mutant for cpnC and performed assays to characterize the phenotype. cpnC- cells had normal growth rates in shaking suspension, but exhibited a slight cytokinesis defect with more multinucleated cells compared to parental cells. When cpnC- cells were starved, they exhibited several developmental defects. Although cpnC- cells were able to from aggregates, they did not form streams. Once aggregated, they formed large ring structures. These rings separated into multiple smaller slugs that developed into smaller fruiting bodies as compared to parental cells. cpnC- cells were less adherent than parental cells to plastic dishes and displayed decreased adherence to 1 um fluorescence beads. The decreased bead adherence of cpnC- cells was still observed after treatment with latrunculin A, suggesting that actin filaments did not contribute to the reduced adhesion. When cells were treated with a protease, bead adherence was no longer significantly different between cpnC- cells and parental cells, suggesting differences in cell surface proteins contributed to the reduced adhesion of cpnC- cells. Western blot analysis of the cell-substratum adhesion protein, SibA, showed a decrease in the expression of SibA when compared to the parental cell strain. The ability of cpnC- cells to aggregate with normal timing, but not form streams suggested that CpnC may play a role in cell-cell adhesion. In contrast, cpnC- cells exhibited an increase in cell-cell adhesion and a corresponding increase in the expression of the cell adhesion protein, CsaA. The complete lack of streaming during aggregation and large ring formation during the mound stage of developing cpnC- cells suggests that CpnC may play a role in cAMP signaling, which could account for the observed changes in adhesion protein expression.

16. Chemical compounds released from Dictyostelids repel plant-parasitic nematodes-2

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We have been studying interactions between Dictyostelia and plant-parasitic nematodes. Plant-parasitic nematodes parasitise many plant species, including several major crop plants, and cause agricultural losses of estimated $80billion annually. Our previous study showed that the secretions of Dictyostelium discoideum can repel root-knot nematodes, one group of plant-parasitic nematode, and protect plant root from infection (Saito YF et al., PLoS ONE 13(9): e0204671, 2018).

In this study, we carried out several experiments to test the repellent effects of extracts from fruiting body of Dictyostelids for other group of plant-parasitic nematodes, root-lesion nematodes Pratylenchus coffeae and P. penetrans. In repellent assays on agar plate, extracts from fruiting body of Dictyostelium discoideum can cause repellent behaviour of root-lesion nematode on petri dish. In infection assay with plant seeding Lotus japonicus, the repellent effect can protect roots from root-lesion nematodes. We further found that fruiting body extracts from Dictyostelia, other than Dictyostelium discoideum, also have repellent effects for root-lesion nematodes.

To identify the nature of repellent activity for plant-parasitic nematodes, we performed biochemical purification of Dictyostelium fruiting body extracts. A couple of purifications with different combinations of chemical purifications, TLC/PLC, HPLC and GC-MS both picked up one repellent compound with molecular mass 208 and molecular formula C8H16O6, which is a non-reducing sugar, Ethyl-a-D-glucoside (aEG). In the plate assay, aEG repels root-knot nematode in a dose dependent manner. The repellent activity of aEG is close to that of potassium nitrate, KNO3, which was previously identified as a repellent for root-knot nematode (Castro CE et al., J. of Nematology 23(4): 409-413, 1991).

This study showed that D. discoideum and other Dictyostelids have repellent activity against root-lesion nematodes as well as root-knot nematodes. In addition, we have identified aEG as a repellent compound for root-knot nematode in Dictyotelium secretions.

17. Clearing the slate: driving developmental progression by mRNA turnover

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Developmental progression requires the change of cell state, enabled by the expression of specific genes and repression of others. Our single cell transcriptomic data show cell state changes during Dictyostelium development are dominated by repressive, rather than activating transitions. Our modelling suggests the importance of large-scale mRNA turnover in removing barriers to developmental progression. We are now testing whether mRNA turnover is constitutive, or induced by developmental signalling, by using single cell imaging of gene expression, in live and fixed cells, to simultaneously measure transcription and mRNA degradation during development. Initial data suggest induced turnover can override continued transcription in remodelling the transcriptome content of differentiating cells.

18. The Polyspondylium pallidum adenylate cyclase A homologs mediate initiation of aggregation, but are not essential for multicellular morphogenesis

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ABSTRACT
In Dictyostelium discoideum adenylate cyclase A is a central component of the protein complex that generates the cAMP oscillations that coordinate aggregation and post-aggregative morphogenesis. Unlike group 4 species like D. discoideum, species in groups 1,2 and 3 do not use cAMP to aggregate, but deletion of cAMP receptors (cARs) or extracellular phosphodiesterase (pdsA) in the group 2 species Polyspondylium pallidum blocks fruiting body formation, suggesting involvement of oscillatory cAMP signalling in post-aggregative morphogenesis. To consolidate these findings we deleted the three adenylate cyclase A genes of P. pallidum individually and in combination. aca1$^-$ cells showed longer and thinner stalks than wild-type cells, aca2$^-$ cells showed delayed formation of secondary sorogens and aca3$^-$ cells formed long aggregation streams, which fragmented to form individual fruiting bodies. The aca1$^-$aca2$^-$ and aca1$^-$aca3$^-$ mutants combined the features of the individual phenotypes. However, aca2$^-$aca3$^-$ and aca1$^-$aca3$^-$aca2$^-$ mutants additionally showed > 24 h delay in aggregation with only few aggregates formed with very long fragmenting streams. Strikingly, the fragments still developed into small branched fruiting bodies with apparently normal stalk and spore cells. Normal aggregation was restored in aca2$^-$aca3$^-$ and aca1$^-$aca3$^-$aca2$^-$ by including 2.5 mM 8Br-cAMP in the agar. These results suggest an essential role for aca1,2 and 3 in PKA activation in early development, but only minor roles in post-aggregative morphogenesis. We provide biochemical evidence that in the aca null mutants, transient cAMP receptor induced cAMP synthesis can also be mediated by adenylate cyclase G.

19. Frequency-dependent predation by a hyper-generalist predatory microbe on multi-species bacterial communities.

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Predation research had long been the remit of behavioural ecologists, focused on the world of the very large. However, this monopoly has been challenged by advances in our microbial sequencing, imaging, and culturing technologies. The ecosystems of the very small have been reimagined as bustling marketplaces, filled with a complexity of competitive and cooperative interactions. In tandem, the amoeba Dictyostelium discoideum is emerging as a model system for studying predation thanks to its hyper-generalist dietary breadth, bacterivorous nature, and the ease of its care and storage. Employing simple cell counting, we tested whether an established principal of macroscopic predation - apostatic selection, or a predator's tendency to over-predate the more common prey type, maintaining prey diversity as a result - might direct D. discoideum's behaviour. Previous work established that D. discoideum has unpredictable effects on the competitive outcomes between different knockouts of the same Pseudomonas species in mixture (Inglis et al. 2018). Here, we assess how D. discoideum (AX4) shapes the competitive dynamics of different bacterial species across a range prey mixtures. With bacterial pairs of similar edibility, D. discoideum appears to interact in frequency-dependent manner, as expected under a regime of apostatic selection. But early results suggest a complex picture in which edibility, prey species, starting prey frequency and predator density interact. Ultimately, our results are likely to better our understanding of how adaptable these organisms are in the face of change, while assessing whether the rules governing predation 'atop' the tree of life may be generalisable across it.

20. Characterisation of peroxisomes in *Dicytostelium*

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Peroxisome is an organelle that is found in most of eukaryotic cells. It is a ubiquitous organelle that is mostly round in shape and which measures about 1 μm. Moreover, this organelle has many significant roles to play in cells, including the beta (β) oxidation of fatty acids and hydroxy peroxide (H2O2) detoxification. It contains many important enzymes including oxidases and catalase, and has several metabolic and non-metabolic functions, depending on its environment and the organisms inside the cells.

However, peroxisome has been almost completely ignored in studies of *Dictyostelium discoideum*. In this study I have been investigating how whether the number of peroxisomes is dynamically regulated in response to different growth conditions. In addition, I have undertaken a proximity labelling approach (BioID) in this research in order to identify the components of peroxisomal import and contents.

21. Nucleocytoplasmic Proteins are Modified by O-fucose in Dictyostelium

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Early in evolution, a gene duplication generated independent lineages of O-GlcNAc-transferase (OGT)-like enzymes, named Secret Agent (Sec) and Spindly (Spy) in Arabidopsis where both are found. Members of the Sec lineage are confirmed OGTs in plants and animals. With their nearly indistinguishable sequences, Spy lineage proteins were assumed to be OGTs until the recent demonstration that Spy sequences in Arabidopsis and the apicomplexan parasite Toxoplasma gondii utilize GDP-Fuc rather than UDP-GlcNAc as donor substrates. Now we show that Spy-like sequences in amoebozoan protists from a distant evolutionary branch, Dictyostelium and Acanthamoeba, are also O-fucosyltransferases (OFTs). The O-Fuc proteins are, as for O-GlcNAc proteins of plants and animals, found in the cytoplasm and nucleus. Their detection in Dictyostelium and confirmation by mass spectrometry were assisted by prediction and disruption of the GDP-fucose transporter (modE locus), which abrogates all fucosylation in the secretory pathway. Capture of O-Fuc proteins with Aleuria aurantia agglutinin and proteomics analysis by nLC/MS identified numerous nucleocytoplasmic O-Fuc proteins in Dictyostelium, including a DNA helicase/transcription factor, a RING/Zn-finger containing protein, and FG-nucleoporins. As for Toxoplasma, knockout of Dictyostelium OFT resulted in a modest growth defect under optimal growth conditions. A comprehensive search for Sec- and Spy-like sequences suggests that both genes were present in the last eukaryotic common ancestor, but that various bacteria, protists and green algae possess just one or the other, certain pathogenic fungi and animals have just OGTs, while red algae and higher plants have both. Our development of new rabbit antibodies specific for fucose-O-Ser and fucose-O-Thr (anti-FOS/T) that do not cross react with fucosylated N- and O-glycans from the secretory pathway promise to facilitate future studies and verify the gene predictions. The high degree of conservation of sites of deleterious mutations in OGTs suggests that these forms of monoglycosylation have a shared role in regulation of responses to stress.

22. Investigating how different PI3-kinase isoforms contribute to macropinocytosis

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Previous work has indicated that different Class I Phosphoinositide 3-Kinase (PI3K) isoforms play different roles during the formation of macropinosomes. This project proposes to unravel the molecular mechanisms of isoform-specific PI3K activities and localisations during the formation and closure of phagocytic and macropinocytic cups in *Dictyostelium discoideum*. It also remains to be understood how exactly a macropinosome is formed, what the specific Class I PI3K roles are during phagocytic and macropinocytic cup formation, and why different isoforms play different contributing roles in these endocytic mechanisms. We hypothesise that it is specific, upstream and downstream interactions that impart the differential roles of PI3K isoforms during phagocytic and macropinocytic cup formation, with domain-specific differences leading to interactions with unique regulator and effector molecules, in turn resulting in differing isoform activities. Due to the high sequence similarity between *D. discoideum* PI3K homologues, we expect these differences to be very small and at the amino acid level, but with a significant effect on protein function. Here we show that PikA (PI3K1)and PikF (PI3K4) do not rescue each other's loss of function, and hence that they possess unique roles in macropinocytic cup formation. We also present preliminary data pointing to domain specificity in the two isoforms, which has been elucidated through the use of hybrid constructs of the two proteins.

23. mTORC1 as a potential regulator of cell fate decisions in *Dictyostelium discoideum*

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Understanding the mechanisms underlying cell fate decisions in response to genetic and environmental factors will help to develop advanced stem cell therapies such as tissue regeneration or replacement of damaged or diseased cells. When *Dictyostelium discoideum* amoebae are starved, vegetatively growing cells aggregate and generate a multicellular organism, which ultimately develops into a fruiting body comprising two major alternative cell types, spores and stalk cells. In mixed aggregates, heterogeneities in genetic background or environmental conditions during growth influence cell fate decisions. Environmental factors (such as nutrient history, intracellular calcium levels and acidic vesicle pH) are well-documented, however the underlying mechanism remains unclear. One potential link between the environmental factors is the activity of a protein kinase complex known as Mechanistic Target of Rapamycin 1 (mTORC1).

mTORC1 is an important activator of cell growth which is regulated by nutrient status and is present on acidic vesicles. In Dictyostelium, TPC2 is a putative Ca2+ channel on membranes of acidic vesicles and tpc2- strains have altered calcium signalling, increased acidic vesicle pH and increased mTORC1 activity (Chang et al., 2020). Consistent with a role for mTORC1, tpc2- cells prefer a stalk cell fate. To investigate whether altering mTORC1 activity can underlie cell fate bias, we have analysed mTORC1 activity in strains with different cell fate preferences and the consequences of both pharmacological inhibition of mTORC1 and genetic modulation of genes encoding mTORC1 regulators, such as AMP-activated protein kinase (AMPK) and Rheb. This work suggests that mTORC1 activity during both growth and development can influence cell fate determination with wide-spread implications for directing stem cells towards defined fates.

Reference: Chang, F.S., Wang, Y., Dmitriev, P., Gross, J., Galione, A., Pears, C., 2020. A two-pore channel protein required for regulating mTORC1 activity on starvation. BMC Biol 18, 8.

24: Identification and validation of substrates for phosphorylation by PKA in *Dictyostelium discoideum*

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PKA is one of the most important protein kinases regulating Dictyostelium multicellular development, but its phosphorylation substrates remain largely undiscovered. In this work, we adopted 2 phosphoproteomics screens to detect the phosphopeptides arisen from PKA phosphorylation in vivo and have identified 12 PKA phosphorylation targets: 4 metabolic enzymes and 8 signal transduction proteins. No gene regulatory/transcription factors were detected, which may be due to their low abundance in Dictyostelium cells. We validated four putative PKA targets in vitro by a thio-phosphorylation reaction, incubating synthetic peptides with a modified PKA (PKA-F413G or AS) and a bulky ATP analogue (6-benzyl-ATPγS). We complemented null mutants of two candidate PKA targets (UgpB and RasGefM) by transformation with wild-type and non-phosphorylatable forms of the target proteins and found that phosphorylation of the proteins was required for complementation and acted to protect the proteins from degradation.

Keywords: PKA, phosphorylation/thio-phosphorylation, protein stability, Dictyostelium.

25: Shaping actin into waves and cups by differential GTPase activity

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Actin polymerisation drives membrane rearrangements during diverse processes such as cell migration, endocytosis and organelle homeostasis. How actin polymerisation is organised in time and space to produce useful work is an important ongoing question. During macropinocytosis, large, actin dependent deformations of the plasma membrane result in the uptake of extracellular fluid, allowing the cell to extract soluble nutrients and detect antigens present in the environment. Lab strains of *Dictyostelium* constitutively perform macropinocytosis, producing stereotypical macropinocytic cups which are organised around patches of PIP3, active ras and active rac. Actin polymerisation must be differentially regulated across this patch to produce protrusive force at the rim of the cup. The King lab has recently demonstrated a differential in the size of the patches of active rac and active ras. This observation has led to a proposed model in which actin polymerisation by the actin nucleating Arp2/3 complex is constrained to an annulus in which rac, but not ras, is active. We hope that further investigation of this annulus model will provide insight into how ras and rac cooperate to organise actin polymerisation.

26. Phosphorylation of the Scar/WAVE complex: a way to tune cell migration.

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The Scar/WAVE complex drives actin polymerization to produce lamellipodia and pseudopodia at the leading edge of migrating cells. This pentameric complex is comprised of Pir121/Sra-1, Nap1, Scar/WAVE, Abi and HSPC300. The detailed mechanisms of Scar/WAVE activation are not well understood. Candidates include its binding to Rac, and phosphorylation by receptor-activated protein kinases such as Erk2. We have therefore investigated the phosphorylation status of Scar/WAVE and Abi, and their importance in the activation of the complex in *Dictyostelium discoideum*.

We find phosphorylation of both Scar/WAVE and Abi is adhesion-stimulated but, surprisingly, phosphorylation of only Abi and not Scar/WAVE is affected by extracellular signalling. Both proteins are phosphorylated at numerous sites in the proline-rich region and do not cause activation of the complex. Instead, Phosphorylation events are consequence of the complex activation. Unphosphorylated Scar/WAVE or Abi increases the lifetime and accumulation of the complex at the leading edge, resulting in longer-lived pseudopodia. Several kinases are involved; ERK2 is not important, but members of the STE20 family control a substantial fraction of the phosphorylation. Hence, our work confirms phosphorylation of the Scar/WAVE complex and is post-activation event to tune the cell migration.