Almost all cells display some form of polarity, and the mechanisms by which this comes about is a fundamental question in cell biology. In many cases, polarity is driven by molecular feedback pathways in a small network of polarity proteins - the PAR network. Whilst many of the key players and interactions in the network have been identified, there is still much to be learnt about how these proteins interact to organise cell polarity, particularly in a quantitative sense.

The \textit{C. elegans} embryo is an attractive model system for studying the design principles of PAR polarity. Work in recent decades suggests that multiple feedback pathways are at play to drive polarity in the \textit{C. elegans} embryo, but some of these pathways are poorly understood. A notable example is a proposed pPAR positive feedback circuit involving the RING-domain protein PAR-2. Such a pathway has been suggested to underlie robust pPAR domain maintenance, even in conditions where spatial information from the aPARs is lost, but this has been poorly understood both from a mechanistic and quantitative point of view. In this project, I used an interdisciplinary approach to study the molecular circuitry of feedback reactions in the PAR network, focussing on PAR-2.

My work began by developing an image analysis pipeline to allow for quantitative analysis of protein distributions from confocal images. With these tools in hand, new experimental possibilities were opened up.

**A mechanistic basis for PAR-2 positive feedback**

A main focus of my project has been aiming to understand the mechanisms that contribute to PAR-2 patterning. To this end, I started by investigating a series of PAR-2 mutants (AxA, C56S, R183-5A, R163A) with known polarity-defective phenotypes. My results suggest that these mutants fail for different reasons, suggesting that a number of processes are at play to drive PAR-2 polarity. Whilst performing this analysis, I also found that wild type PAR-2 deviates from expected linear membrane binding kinetics, which is the first direct evidence for a positive feedback reaction. I also found that this nonlinearity is lost in RING domain mutants, suggesting a RING-dependent mechanism.

Next, I decided to investigate the mechanisms of RING domain action, which were previously unknown. My first line of inquiry involved a hypothesis that PAR-2 might be able to autoubiquitinate, in line with commonly established roles for RING domains. Whilst my data cannot definitively rule out such a role, my data points to an alternative mechanism. Using in vitro and in vivo experiments, I have shown that the RING domain of PAR-2 can dimerise, and that, in vivo, this specifically occurs when local concentrations are enriched by membrane binding. Additionally, by comparison to thermodynamically constrained mathematical models, I have shown that this reaction can explain the observed positive feedback behaviour, and the difference in membrane affinity between wild type and RING mutant PAR-2.

Whilst the PAR-2 RING has long been known to be important, this is the first direct evidence supporting a mechanism of action in vivo. Additionally, whilst the ability of RING domains to dimerise is well established, a role for this reaction in driving positive feedback on a membrane association reaction is, to the best of my knowledge, a novel function for a RING domain.

**Dimer affinity is optimised for positive feedback and membrane specificity**

Typically, it is thought that increasing the electrostatic charge on a protein should increase its specificity for the highly charged plasma membrane \citep{Yeung2008}. Following this logic, dimerisation, which should effectively double the net charge per molecule, is expected to be productive for strong plasma membrane association. However, my results for PAR-2 show that both disrupting dimerisaton and enhancing dimerisation can weaken localisation at the plasma membrane (\cref{fig:mutant\_comparison\_for\_discussion}), which is counter to predictions from equilibrium models.

I propose the model presented in fig x. Pre-symmetry breaking, the system is held in an equilibrium state in which PAR-2 is kept off the plasma membrane, whilst low-affinity internal membrane association is permitted. Following symmetry breaking, the posterior plasma membrane becomes accessible, via flow-driven aPAR clearance and posterior protection of PAR-2 by microtubules, and the system transitions towards a new equilibrium state. Membrane-specific dimerisation, driven by the intermediate-strength RING domain, leads to a positive feedback reaction which amplifies posterior plasma membrane association (fig xA).

In this scheme, both increasing dimerisation strength and decreasing dimerisation strength can weaken localisation at the plasma membrane, but this occurs for different reasons (fig xB). At low dimerisation strengths (e.g L109R), protein is unable to dimerise at the membrane, and therefore unable to partake in a positive feedback reaction to stabilise the high-affinity dimeric state. However, at the other end of the scale, strong dimerisation (GCN4), which slows membrane exchange timescales, can cause effective entrapment on internal membranes prior to symmetry breaking, which can sequester PAR-2 away from the plasma membrane. Wild type PAR-2 dimerisation, mediated by an intermediate strength RING domain, appears to put the protein in a sweet spot that allows it to bind strongly to the plasma membrane, but avoid kinetic entrapment on internal membranes.

Whilst my data makes a good case that the RING-domain of PAR-2 drives intermediate-strength dimerisation, and my theoretical models have been able to account for a number of maintenance-phase behaviours of wild-type and mutant PAR-2, several aspects of the model could be strengthened with further experimental work.

Firstly, my conclusions are largely based on analysis of single timepoints at maintenance phase. To fully validate the model, more work is required to analyse the temporal dynamics of polarity onset in different conditions, including quantitative assessment of pre-symmetry breaking states.

Secondly, whilst I have been able to establish that wild-type PAR-2 dimerises at the membrane in vivo, my current data does not give any direct quantitative information about the degree of dimerisation, which would be useful for comparison to the thermodynamic model. In vivo imaging techniques such as FLIM (fluorescence lifetime imaging)-based FRET (Forster resonance energy transfer), which uses fluorescence lifetime measurements to monitor the transfer of energy between two fluorophores in close proximity, could be used to directly monitor the degree of dimerisation at the membrane (and in the cytoplasm). Furthermore, super-resolution microscopy techniques, such as SIM (structured illumination microscopy) may allow us to resolve and quantify distinct monomeric and dimeric populations at the membrane.

Thirdly, the model relies heavily on the claim that strengthening dimerisation reduces membrane unbinding rates. Whilst our model gives good theoretical backing to this claim, we have yet to demonstrate this experimentally. Techniques such as FRAP (ref) and smPRESS (ref) have previously been used to explore the membrane exchange kinetics of PARs, including PAR-2 itself, although each method comes with caveats and success in our hands has been limited so far.

**Comparison to other PAR proteins**

As discussed in the introduction, membrane binding strategies vary between different PAR proteins. Some PARs, such as PAR-3 and CDC-42, rely mainly on binding to specific phospholipids enriched at the plasma membrane, whereas others, like PAR-2 and PAR-1, are thought to rely mainly on electrostatic interactions. In the former case, plasma membrane specificity is an obvious outcome of lipid specificity. In the latter case, whilst explanations for plasma membrane specificity have been put forward, my work suggests that additional considerations are needed in the context of dynamically changing systems.

Interestingly, whilst this has not been proven, PAR-1 cytoplasmic gradients have been speculated to come about through an interaction with endoplasmic reticulum (ER) membranes \citep{Folkmann2019}. Whilst PAR-1 is not thought to dimerise, it is interesting to speculate whether constitutive PAR-1 dimerisation might shift its balance towards stronger ER localisation.

Talk about other non-C elegans proteins here? Miranda, Numb

i.e. the fact that they have polybasic domains and bind to anionic phospholipids

**Contributions of dimerisation-driven feedback to bistable patterning**

In chapter x, I investigated the functional importance of the PAR-2 dimerisation reaction in patterning models, using a simple two species model with linear antagonism terms. My analysis shows that dimerisation coupled to mutual antagonism can be sufficient to generate stable patterns without other sources of nonlinearity, provided that dimers be, at least partially, resistant to antagonism compared to monomers. Results in chapter 7 suggest that dimers might be partially resistant to antagonism by PKC-3, but more work is needed to confirm this. Mathematical models would also be useful here to investigate the potential links between dimerisation and antagonism, and the requirements for differential antagonism, but this is yet to be explored.

In reality, the system likely contains multiple sources of nonlinear behaviour, and so likely does not rely solely on any one source of nonlinearity to generate bistability. Nevertheless, having multiple sources of nonlinear feedback is expected to enhance the overall robustness of the system \citep{Chau2012}.

Relating this back to original observations that PAR-2 can remain polarised in conditions with uniform aPAR, many questions are still unanswered. If dimers are resistant to antagonism, for whatever reason, then dimerisation would suggest an ability of PAR-2 to resist PKC-3 when highly concentrated. However, whilst models in these regimes can show a nonlinear relationship between kinase concentrations and antagonism, this relationship displays no signs of bistability (\cref{fig:model\_antagonism\_effective\_exponent}), so cannot generate a spatial asymmetry without additional sources of spatial information. It is possible that additional pPARs, such as LGL-1 and CHIN-1 might act to locally inhibit PKC-3 activity. However, were this to be the case, it is unclear why the PAR-2 point mutants investigated would be unable to polarise in these conditions. Perhaps there might be a complex interaction between PAR-2 and LGL-1/CHIN-1 that we are not yet accounting for. Alternatively, PAR-2 itself may act to locally inhibit PKC-3 activity. How this might come about, and whether this could create bistable behaviour, needs further investigation.

**Additional roles of PAR-2**

Probably the most notable role for PAR-2 is as a scaffold for the pPAR kinase PAR-1. Central to the mutual antagonism model, membrane recruitment of PAR-1 by PAR-2 is responsible for locally excluding aPARs through a phosphorylation reaction from PAR-1 to PAR-3. This effectively makes up one of the antagonism terms in the simple patterning models that I have used, in which PAR-1 is not explicitly included.

However, the reaction between PAR-1 and PAR-2 likely has additional downstream roles. Since PAR-1 directly regulates downstream fate determinants, PAR-2 is expected to affect this pathway by regulating the asymmetry of PAR-1. Surprisingly, however, PAR-2 appears dispensable for proper segregation of fate determinants in the zygote \citep{Kemphues1988, Boyd1996}. Instead, it is thought that PAR-1 asymmetries in the cytoplasm, rather than at the cortex, are responsible for segregating fate determinants \citep{Folkmann2019}, although it is still not understood how these cytoplasmic asymmetries come about. Notably, however, localisation of fate determinants in PAR-2 mutants is impaired at later developmental stages in embryos \citep{Kemphues1988}. Thus, a primary function of the PAR-1/PAR-2 interaction may be to ensure that PAR-1 is properly segregated and enriched through the developing P-lineage, so that downstream signalling can continue in, and be restricted to, the P-lineage. The ability for PAR-2, and thus PAR-1, to reach high cortical concentrations within polarity domains would likely help this process.

In addition, PAR-2 is thought to have a number of other roles that are not carried out through PAR-1. One such role is the ability to restrict posterior-directed cortical flows at maintenance phase \citep{Munro2004, Beatty2010}. In addition, recent work from the Goehring lab (Rodrigues et al., in prep) also suggests a role for PAR-2 in regulating spindle pulling forces, although the role that this plays in spindle positioning is unclear. The ability to reach high cortical concentrations through a positive feedback circuit may be important for both of these actions.

We do not know the mechanisms by which PAR-2 carries out these downstream roles. The only other structured domain in PAR-2 is a C-terminal ATP binding domain. However, removal of this domain has been shown to have no phenotype \citep{Hao2006}, so the role for this domain is unclear. Perhaps these roles could also be mediated by the RING domain, perhaps through a ubiquitination pathway. Since I see no observable phenotype in the linchpin (K94A) mutant, my work casts doubt on this, although I have not characterised the behaviour of this mutant in non-wild type backgrounds, so there could be some disruption that I fail to see in wild type backgrounds. This could be interesting to explore, with the caveat that the linchpin is not a universally conserved mechanism, and so is not guaranteed to disrupt ubiquitination activity if there is any.

Given the many roles of PAR-2, I was surprised to see that my dimer interface mutant (L109R), in which membrane affinity is strongly reduced, displays no signs of sterility or embryonic lethality. On the other hand, C56S mutation, which has a stronger effect on membrane affinity, leads to partial sterility and embryonic lethality. The reason for the difference in affinity between these mutants is unclear, but I suspect that the additional loss in affinity in C56S is a result of domain unfolding, which may interfere with membrane binding. Recent work from the Goehring lab (Rodrigues et al., in prep) shows that embryos are able to withstand large (approximately 50\%) reductions in PAR-2 cortical concentrations before significant defects are observed, indicating that the system likely contains multiple sources of robustness. It may be that, whilst C56S pushes worms below this threshold, L109R worms are just above. In this case, I would expect that PAR-2 L109R worms should be relatively sensitive to small reductions in protein amounts. Non-wild-type backgrounds such as \textit{lgl-1} mutants, which are usually viable with wild type PAR-2, may also show defects when combined with PAR-2(L109R).

**Comparisons to other polarity systems**

Where did PAR-2 come from?

Questions remain about the evolutionary origins of PAR-2

**Summary**

Overall, the results of this project enhance our understanding of the role of PAR-2 in PAR polarity maintenance, particularly with regard to the RING domain of the protein. Taken together, my results reveal that a concentration-dependent dimerisation reaction drives a positive feedback pathway, which enhances membrane concentrations and stabilises PAR-2 domains. My work also reveals a key role for an optimisation of dimerisation strength at intermediate levels. Reducing dimerisation energy below this level results in a loss of positive feedback, whilst enhancing dimerisation energy leads to kinetic trapping of PAR-2 on internal membranes, both of which can compromise PAR-2 domain integrity. There are still many unknowns about the roles of PAR-2 in cell polarity, such the nature of its relationship with PKC-3 and the mechanisms by which it carries out its downstream roles, as well as its evolutionary origin, and more work will be required to enhance our understanding in these areas.