**Discussion**

Almost all cells display some form of polarity, and the mechanisms by which this comes about is one of the fundamental questions 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 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 may be at play to drive polarity in the 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**

My main focus 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 with known polarity defective phenotypes. My results suggest that these mutants fail for different reasons, suggesting that a multitude of processes are at play to support PAR-2 polarity. Additionally, I found that wild type PAR-2 deviates from expected linear membrane binding kinetics, indicative of a positive feedback reaction, and that this is lost in RING mutants.

Given that the RING mutant phenotype is so striking, and that the mechanistic role of the RING domain was previously unknown, I decided to push this further and pursue a number of hypotheses. 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, and the different membrane binding behaviour of wild type vs 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.

**Functional importance of RING-driven membrane association**

**Patterning**

I next investigated the functional importance of this reaction in mathematical models. 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, 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 requirements for differential antagonism, but this is yet to be explored.

In reality, the system probably contains multiple sources of nonlinear behaviour, and so does not rely 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 (Chau).

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 may provide an explanation for the ability of PAR-2 to resist PKC-3 when highly concentrated. However, whilst in these regimes the relationship between kinase concentrations and antagonism can be nonlinear, it displays no signs of bistability (fig x), so this 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 PAR-2 point mutants would completely obstruct the ability to polarise. Perhaps there is 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. Notably, my model is in contrast to previous claims that self-recruitment of PAR-2 might allow PAR-2 domains to `self-stabilise’ in the face of uniform antagonism (REFS). I propose that this view be reconsidered, and that researchers focus on other potential explanations for this behaviour.

**Downstream roles of PAR-2**

Perhaps the most notable role for PAR-2 is as a scaffold for PAR-1. Central to the mutual antagonism model, recruitment of PAR-1 by PAR-2, means that PAR-1 can act to locally exclude PAR-3. This effectively makes up the antagonism terms in the simple PAR models that I have used in this thesis, in which PAR-1 is not explicitly included.

However, the reaction between PAR-1 and PAR-2 may also have other roles. PAR-1 affects downstream fate determinants, and so by altering PAR-1 localisation, the membrane affinity is expected to be important. Surprisingly, however, PAR-2 appears dispensable for proper segregation of fate determinants in the zygote. Whilst absent from the cortex in zygotes lacking PAR-2, PAR-1 is still able to maintain a cytoplasmic gradient (REF) and set up normal MEX-5 asymmetries. Notably, however, localisation of fate determinants is impaired at later stages in PAR-2 mutant embryos (REF). Thus, a primary function of the PAR-1/PAR-2 interaction may be to ensure that PAR-1 is segregated and enriched through the germ line, so that downstream signalling can continue in, and be restricted to, the developing P-lineage. The ability for PAR-2, and thus PAR-1, to reach high cortical concentrations 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. In addition, Rodrigues also reports 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 mechanism 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 phenotypes, so the role for this domain is unclear. Perhaps this could also be mediated by the RING domain, perhaps through ubiquitination pathways. Since I see no observable phenotypes in the linchpin 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 L109R mutants, in which membrane affinity is strongly reduced, display no signs of sterility and lethality. On the other hand, C56S, which has a stronger effect on membrane affinity, does display signs of both sterility and lethality. The reason for the difference in affinity between these lines 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 (~50%) losses in PAR-2 before significant defects are observed, indicating that the system likely harbours multiple sources of robustness. It may be that, whilst C56S pushes worms below this threshold, L109R worms are just above. In this case, I expect that PAR-2 L109R worms should be relatively sensitive to small reductions in protein amounts. Non-wild-type backgrounds such as LGL-1 mutants and nop-1 mutants (reduced cortical flow), which are usually viable with wild type PAR-2, may also show defects when combined with PAR-2(L109R).

**Summary**

Overall, the results of this project enhance our understanding of the roles of PAR-2 in PAR polarity maintenance, particularly with regard to the RING domain of the protein. Taken together, my results suggest that a concentration-dependent dimerisation reaction drives stable membrane association of PAR-2 and constitutes a positive feedback pathway that can contribute to stable pattern maintenance. Currently there are still many unknowns about the roles of PAR-2, and the pPARs more generally, in self-organising polarity, and more work will be required to fully understand this. My results also raise some interesting new ideas about the interplay between dimerisation and membrane specificity, which may be interesting for further study.