**Introduction**

**The PAR-2 polarity pathway**

As previously discussed, PAR-2 is able to initiate polarity in the absence of cortical flows through an interaction with microtubules. This interaction protects PAR-2 from phosphorylation by PKC-3, which leads to localised recruitment of PAR-2 to the cortex at the posterior pole. Cortical PAR-2 then recruits PAR-1 from the cytoplasm, which antagonises aPARs through phosphorylation of PAR-3. According to a simple mutual antagonism model, if a threshold concentration of PAR-2/PAR-1 is reached, self-organisation can be initiated, resulting in the formation of a normal polarised PAR pattern (fig x).

FIGURE: mt symmetry breaking. supra vs sub-threshold recruitment

Using an in vitro microtubule pelleting assay, along with analysis of truncation mutants, the authors were able to identify regions of the protein responsible for microtubule binding. Two mutants to basic residues within these regions (R163A and R183-5A) were found to reduce the level of microtubule binding in vitro, and prevent symmetry breaking in no-flow conditions in vivo.

Strikingly, and in contrast to predictions from the mutual antagonism model, it has been shown that formation of a stable PAR-2 domain in these conditions doesn’t require aPARs to be cleared from the posterior. In par-1 mutant conditions, or with a par-3 non phosphorylatable mutant, PAR-2 domains are able to be initiated, and spread, without corresponding clearance of aPARs, leading to spatial overlap of aPARs and pPARs in the posterior. The fact that PAR-2 can polarise without spatial information from the aPARs implies that additional positive feedback mechanisms must exist that contribute to PAR-2 polarity (fig x). Specifically, the fact that PAR-2 domains can overlap with PKC-3 implies that feedback mechanisms must exists that allow cortically enriched PAR-2 to resist or counteract antagonism from PKC-3 in the posterior. Whilst we do not have a complete picture of these feedback pathways, some insights shed light on potential mechanisms.

FIGURE: schematic of polarity regimes

**Self-recruitment**

Interestingly, Motegi observed that the microtubule mutants, which are usually unable to polarise in the presence of uniform aPAR (no flow, S950A) can do so when wild type PAR-2 is present. This has led to proposals that PAR-2 may have the ability to ‘self-recruit’, meaning that cortical PAR-2 molecules may recruit additional cytoplasmic PAR-2 molecules to the cortex. In line with this, PAR-2 has been shown to self-associate in in vitro pull-down assays 2 \citep{Motegi2011, Arata2016}.

Arata expanded on this by showing that PAR-2 exists as oligomers at the cortex. By TIRF imaging, the authors were able to resolve distinct PAR-2 particles of varying size at the cortex, which showed a slight asymmetry towards larger oligomers at the posterior. Membrane lifetime was found to vary across the cell according to oligomer size and local PKC-3 concentration, indicating that oligomerisation can increase, and phosphorylation decrease, the stability of membrane binding. They also found an asymmetry in the membrane association rate, highest in the posterior of polarised cells, which they suggest could be due to direct recruitment of cytoplasmic PAR-2 into cortical oligomers.

Self-recruitment may lead to a positive feedback reaction, whereby cytoplasmic PAR-2 is preferentially recruited to regions of high cortical PAR-2. Such a reaction may allow the PAR-2 domain to independently grow and self-stabilise. However, the extent to which this reaction impacts the membrane binding kinetics of PAR-2 is not understood, and we lack quantitative data to assess the strength and significance of this feedback.

**RING domain**

Studies of mutant PAR-2 alleles have demonstrated an important role for the N-terminal RING domain in establishing strong PAR-2 domains. Under normal conditions, RING mutant PAR-2 can form a posterior polarity domain following aPAR advection, but this domain is weakly concentrated compared to wild type PAR-2. Following cessation of flows in the maintenance phase of polarity, aPARs spread back towards the posterior, causing the PAR-2 domain to be largely cleared by the time of cytokinesis (Hao). This implies that the C56S domain lacks the fundamental properties of wild type PAR-2 domains that allow them to overcome antagonism from PKC-3. In no flow conditions PAR-2 C56S remains entirely cytoplasmic and unable to trigger formation of a domain (Motegi). Whilst it’s clear that the RING plays a significant role in organising the PAR-2 domain, insight into the mechanisms of RING domain action is lacking. The extent to which the RING phenotype is a consequence of intrinsically lower membrane affinity, failures in the self-recruitment pathway described above, or a represents a specific role for the RING domain in counteracting PKC-3 (perhaps by making the protein a poorer substrate) is unclear.

**Substrate-kinase interaction**

Unpublished work by Florent Peglion (Goehring lab) has shown that a mutant PAR-2 allele with a weakened PKC-3 recognition motif, whist able to polarise relatively normally in wild type conditions, is unable to drive polarity in no flow conditions. A possible interpretation of this is that the ability for PAR-2 to resist PKC-3 might, perhaps counterintuitively, rely on tight association between PAR-2 and the kinase. Whilst intermediate substrate affinity is often compatible with efficient enzymatic activity, high substrate affinity can lead to a stable interaction between a kinase and substrate which blocks enzymatic activity. PAR-3, which has a similar PKC-3 recognition site, can act as either a substrate or an inhibitor of PKC-3 depending on affinity (Soriano). In the case of PAR-2, regulation of binding affinity to PKC-3 may underlie its ability to resist antagonism in some contexts, whilst being sensitive in other contexts. Work to uncover the nature of the PAR-2/PKC-3 interaction is ongoing in the Goehring lab.

**Outlook**

Overall, this work paints a picture of PAR-2 polarity that extends beyond the mutual antagonism model. Whilst under normal conditions, advective transport and mutual antagonism may dominate the patterning procedure, the secondary microtubule-dependent pathway appears to rely on different feedback reactions intrinsic to PAR-2. Following initial recruitment to the cortex by microtubules, self-recruitment of PAR-2 molecules from the cytoplasm enhances cortical PAR-2 concentrations. Diffusive spread, along with the ability to resist PKC-3 when highly concentrated allows PAR-2 to spread anteriorly and rapidly invade cortical space occupied by aPAR. At the same time, cortical PAR-2 recruits PAR-1 from the cytoplasm. By phosphorylating PAR-3, PAR-1 eventually clears aPARs from the posterior, restricting them to the anterior.

However, our understanding of this process is far from complete. Primarily, whilst feedback pathways have been proposed, we lack insight into the quantitative nature of these pathways, which limits our ability to understand the system with computer models. Furthermore, we lack good insight into the quantitative characteristics of wild type PAR-2 and the various mutant alleles, which complicates interpretation of phenotypes and limits our abilities to build computer models. Recent advances in techniques for in vivo quantification, described in the previous chapter, overcome some of these barriers and should enhance our ability to obtain accurate quantitative data.

Additionally, previous studies are complicated by their reliance on transgenic lines. As expression of transgenes is not under normal regulatory control, and the location of transgene insertions in the genome varies from line to line, protein levels are often not comparable to endogenous protein levels, and can strongly differ between lines. This is a potential concern, as the ability for PAR-2 to polarise in no-flow conditions is sensitive to small changes in PAR-2 amounts (Rodrigues). Recent advances in CRISPR technologies mean that we can now tag and monitor endogenous PAR-2 with relative ease, which may control for this potentially confounding factor.

**Quantitative characterisation of PAR-2 mutant alleles**

With this in mind, my first aim was to perform quantitative analysis of the membrane binding behaviour of PAR-2 and the mutant alleles described previously which have reported polarity-defective phenotypes (R163A, R183-5A, AxA and C56S). By CRISPR to an endogenously tagged GFP::PAR-2 line, we created a series of point mutants at the endogenous locus (fig xA). In order to separate intrinsic and PKC-3 dependent behaviours, I crossed each line to a line expressing the par-3 it71 allele (ref), in which PAR-3 is <not expressed?> and PKC-3 is held off the cortex and inactive. Under these conditions, PAR-2 binds uniformly to the cortex (ref). Using the protocol described in the previous section involving autofluorescence correction and <>, I <quantified>. In fig x I present membrane to cytoplasmic ratios, a measure of membrane affinity. Fig x shows full quantification results, including cytoplasmic concentrations, membrane concentrations and total protein levels.

As expected, whilst PAR-2 AxA behaves differently to wild type in polarised cells, owing to its weaker association with PKC-3, membrane affinity in uniform cells is equal to wild type, suggesting that the mutant harbours no intrinsic defect in membrane binding.

PAR-2 C56S, by contrast, has a membrane affinity greatly reduced compared to wild type. This is the case even in par-3 mutants, suggesting that the localisation defect is intrinsic to PAR-2, rather than via hypersensitivity to PKC-3 as has previously been suggested (ref). Overall protein levels are also slightly reduced compared to wild type (fig x). This is in contrast to previous results using transgenic lines which showed higher levels in the mutant, and was used to argue for auto-regulation of degradation via autoubiquitination. The reason for this discrepancy is unclear, but I believe that the small reduction compared to wild type may relate to protein instability as a result of the unfolded RING domain.

Surprisingly, I also found that the microtubule mutants have a weaker membrane affinity compared to wild type PAR-2. This is independent of aPARs, suggesting that the defect is intrinsic to PAR-2. Such a defect has previously not been reported in previous studies using this line (refs). These mutants also have a mild overexpression phenotype (fig x). As a result, membrane concentrations are similar to wild type concentrations despite the reduction in membrane to cytoplasmic ratio.

**RING domain promotes positive feedback to achieve optimal membrane affinity**

Another striking feature of the data that I observed is that the membrane affinity of wild type PAR-2 appears higher in the posterior domain of polarised cells than in uniform cells. Whilst membrane concentrations are expected to be higher in polarised cells due to the increase in concentration of the cytoplasmic pool, membrane affinities are not expected to change. In other words, membrane and cytoplasmic concentrations should follow a linear trajectory (fig x).

This led me to hypothesise that there might be a nonlinear relationship between cytoplasmic and membrane concentrations. Positive feedback, where PAR-2 is promoting its own recruitment to the membrane, may lead to a nonlinear relationship between membrane and cytoplasmic concentrations if feedback is sufficiently strong. The expected shape of the relationship depends on the strength and form of positive feedback (fig x). Linear feedback <>. However, under some parameter regimes, nonlinear feedback may lead to bistability, whereby multiple membrane concentrations can be supported within a range of cytoplasmic concentrations (fig x). <Wave pinning>.

The quantification pipeline means that it’s possible to accurately measure membrane and cytoplasmic concentrations on an embryo-by-embryo basis, meaning that dose response studies can be performed (Rodrigues). To test for the existence of positive feedback intrinsic to PAR-2, I performed an assay using par-3 mutant worms where I performed an RNAi rundown to titrate protein levels, and quantified membrane and cytoplasmic concentrations on an embryo-by-embryo basis.

The results confirm that PAR-2 membrane binding doesn't follow strict linear membrane binding kinetics (fig x). Instead, a nonlinearity appears to be present whereby membrane affinities are higher at higher dosage levels. The shape of the relationship implies that PAR-2 is feeding back onto its own membrane binding (fig x), but there is no evidence for any discontinuities that would imply bistable behaviour.

Interestingly, unlike wild type PAR-2, the membrane affinity of C56S is the same in uniform and polarised systems despite large changes in membrane and cytoplasmic concentrations. This suggests that the mechanism of positive feedback may be RING domain dependent. To test this, I performed the same assay using the C56S mutant line. The results (fig x orange) show that, whilst there may be mild nonlinear behaviour, this is greatly reduced compared to wild type. Overall, I conclude that the RING domain of PAR-2 promotes positive feedback to achieve optimal membrane affinity.