**Structure:**

* **Previous work shows ability to maintain invasion of aPARs. Unclear whether this is stable state, or just represents slow kinetics. Models: is this resistance, or ability to drive polarity?**
* **We’ve extended this by showing that PAR-2 drives polarity**
* **Various mutants are defective at this. Why?**
* **In case of C56S, seems to relate to affinity**
* **OPTIONAL: kinetics indicative of positive feedback. Disrupted in the case of C56S**

**Alternative structure:**

* **Par-2 mutants can’t break symmetry in no-flow conditions**
* **Quantitative analysis of mutants**
* **Polarity even with uniform aPAR**
* **Symmetry breaking behaviour implies intrinsic positive feedback**

**PAR-2 polarity in systems with uniform aPAR**

Also looked for similar behaviour in a mutant of par-3 designed to prevent phosphorylation

In vitro assays confirm that mutation to this single site prevents phosphorylation

Previously only looked at in bombarded lines

Introduced it by CRISPR at the endogenous site in a dual-labelled parent line

See similar behaviour to par-1 mutant

This line will be favoured for further studies as it is considerably less sick

**Symmetry breaking against uniform aPAR**

The polarity phenotypes described above are unusual and not seen in normal polarity conditions

Suggests there might be an alternative mode of symmetry breaking

Took timelapse movies of par-3 s950a embryos with mlc4 RNAi

Beginning with uniform cortical par-6 and par-2 mostly cytoplasmic

Localisation of cortical par-2 at the midcell appears to coincide with a local clearance of PAR-6, suggesting that this is due to a local release of inhibition

Interestingly, however, this local clearance of PAR-6 appears to be transient, and PAR-6 becomes largely uniform again after a few minutes

Despite this, the PAR-2 domain continues to grow in concentration and size, eventually able to stabilise a full domain against uniform aPAR

(In this case, we can also see a smaller domain at the embryo posterior, which may represent normal microtubule dependent symmetry breaking.)

Therefore, whilst local PAR-6 clearance appears to be the cause for initial PAR-2 loading, in line with the mutual antagonism model, growth of the domain can proceed even without aPAR clearance. This suggests that there may be mechanisms of intrinsic positive feedback driving PAR-2 localisation that doesn’t rely on local aPAR clearance.

**Polarity phenotypes in PAR-2 mutants**

Mutants to the putative microtubule binding sites have already been described to disrupt symmetry breaking in no-flow regimes.

However, this has not been tested in CRISPR lines

The results show, as expected, that PAR-2 fails to establish polarity domains in these conditions.

I extended this analysis to some PAR-2 mutants that have previously been described, but not characterised in these conditions.

**Quantitative characterisation of PAR-2 mutants**

To help to understand the phenotypes presented above, I performed full quantitative analysis on this set of mutants

**Microtubule mutants**

In both mutants membrane to cytoplasmic ratios are significantly lower than wild type, with PAR-2 (183-5A) showing the strongest effect. Despite this, domain concentrations are similar to wild type. This can be attributed to a higher overall level of protein in these cells, giving an increased cytoplasmic concentration of PAR-2.

**AxA**

PAR-2 (AxA) binds to the anterior membrane.

The posterior membrane to cytoplasmic ratio is lower than wild type, but in cells lacking aPAR the mutant is identical to wild type

**C56S**

C56S has a considerably lower affinity both in polarised and uniform conditions.

Overall protein levels are slightly lower than wild type. This is in contrast to previous studies, which showed that RING mutant alleles are overexpressed, leading the authors to hypothesise that RING dependent autoubiquitination may play a role in downregulating protein levels. <reason for discrepancy?>. Compatible with this, other mutant alleles in their study with an intact RING domain also showed variable expression levels.

Interestingly, unlike other alleles where affinity is higher in polarised compared to uniform cells, this doesn’t appear to be the case for C56S, where affinity is similar in the two regimes.

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

Transition to section\\

To test for evidence of feedback, I performed an assay where I titrated the level of PAR-2 by RNAi (in a par-3 mutant system) and quantified the membrane and cytoplasmic concentrations in the different embryos (similar to the assay performed previously for PH). Whilst a protein without feedback is expected to follow a linear relationship, a protein feeding back on its own membrane recruitment may instead be expected to have a higher membrane affinity the more total protein there is in the system, giving a nonlinear relationship between membrane and cytoplasmic concentrations. In the case of PAR-2, the assay quite clearly shows that there isn't a straightforward linear relationship between membrane and cytoplasm, with a positive uptick that's characteristic of positive feedback (\cref{fig:rundown\_vs\_c56s}).\\

On the other hand, mutant PAR-2 (C56S), as well as following a lower affinity trajectory, appears to have a more linear relationship between cytoplasm and membrane, more in line with a protein that's exchanging on and off the membrane without feedback. This suggests that the mechanisms of positive feedback, which appear to contribute to the strong membrane binding kinetics of PAR-2, may be RING domain dependent.

**Discussion**