**2. IDENTIFYING CORE PATTERNING BEHAVIOURS OF PAR-2**

*Aims:*

* *Recapitulate old findings that PAR-2 can polarise with uniform aPAR, using CRISPR lines instead of bombarded lines*
* *Explore the molecular/genetic determinants of this state, including the potential role for other pPAR proteins, and the requirement for intact PAR-2*

1. **PAR-2 robustly polarises in systems with uniform aPAR**

*Mutant conditions with broken pPAR to aPAR antagonism (par-1 b274, par-3 s950a) show largely normal polarity in the early embryo and full viability (in the case of par-3 s950a), in contrast to predictions from the mutual antagonism model. Two features are notable:*

* *aPARs do not spread into the posterior as much as expected (less than in old data from bombarded lines)*
* *Overlap between aPARs and pPARs at many stages in the early embryo*

*Disrupting cortical flow in these mutant backgrounds demonstrates that PAR-2 can robustly break symmetry and maintain polarity against a uniform aPAR background:*

* *par-1 (b274) + mlc-4 RNAi*
* *par-3 (s950a) + mlc-4 RNAi*
* *par-3 (s950a) + nmy-2 ts*

*Figures:*

* *Timelapses of par6/2 polarity in wt vs par-1 b247 vs par-3 s950a (+ kymographs to clearly show overlap in latter two)*
* *Viability, sterility, 2-cell asymmetry of wt vs par-3 s950a vs par-1 b247*
* *Timelapses + kymographs in no-flow conditions (+ controls)*
* *Bar/pie charts showing consistency of symmetry breaking in uniform aPAR conditions, location of PAR-2 domains (midcell vs maternal vs paternal vs combinations)*

1. **The role of LGL-1 and CHIN-1 in PAR-2 domain maintenance**

*Assessing the potential role for LGL-1 and CHIN-1 in the maintenance of PAR-2 domains in uniform aPAR conditions. Do these proteins colocalise with PAR-2, and does their removal by RNAi have any impact on PAR-2 polarity? These experiments still need to be done. LGL-1 line is extremely dim and difficult to judge. CHIN-1 GFP is brighter, and crosses using CHIN-1 GFP line are in progress.*

*Figures:*

* *Pending results, but likely similar to above section, looking at LGL-1/CHIN-1 with PAR-6 or PAR-2, or PAR-2/6 in lgl-1 and/or chin-1 RNAi conditions*

1. **Polarisation requires functional PAR-2**

*Resistance appears to be PAR-2 concentration dependent (i.e. weak domains do not resist aPARs at all)*

*- PAR-2 rundown in par-3 (s950a) + nmy-2 ts line*

*Mutations that disrupt PAR-2 fail to polarise in these conditions:*

* *RING domain mutants (e.g. C56S)*
* *‘Microtubule binding site’ mutants (e.g. R183-5A). NB the exact role of these sites is controversial, and may be unrelated to microtubules*
* *PKC-3 binding site mutants (e.g. AxA)*

*These mutants are characterised by low/partial sterility, which is (/may be) increased when combined with par-3 s950a (I need to check this for some mutants)*

*Figures:*

* *Images of wt vs par-2 mutants in wt vs mlc-4 background*
* *Viability, sterility, 2-cell asymmetry of wt vs par-2 mutants vs par-2 mutants + s950a*

1. **Robust polarity implies the existence of additional feedback reactions**

*Polarity in the mutual antagonism model does not work if aPARs cannot be antagonised, as bistability is lost. However, polarity can be restored through an additional positive feedback reaction on pPARs, provided that this reaction is sufficiently nonlinear to support bistability.*

*Figures:*

* *Cytoplasm vs membrane relationship for no positive feedback vs monostable positive feedback vs bistable positive feedback models (showing bistability in the latter case only)*

1. **Conclusions**

* *Polarity is not completely lost when mutual antagonism is broken, highlighting independence of aPARs, and ability of PAR-2 to resist antagonism. Compare to old data*
* *LGL-1 and CHIN-1 may or may not contribute PAR-2 polarity in uniform aPAR conditions. This is important as it will determine whether the feedback maintaining PAR-2 polarity is intrinsic or extrinsic to PAR-2*
* *RING domain clearly important. Surprising that the line is partially viable, compared to old data where RING mutants don’t rescue. Unclear what its role is, whether defects are purely related to membrane affinity or whether something is fundamentally different*
* *Altogether, these results are incompatible with the mutual antagonism model, and imply the existence of additional feedback reactions in the network. It is therefore worth investigating PAR-2 behaviour more quantitatively to determine how this might work*

**3. A PIPELINE FOR QUANTIFICATION OF MEMBRANE AND CYTOPLASMIC PROTEIN CONCENTRATIONS**

*Aims:*

* *Develop and validate an approach to quantify underlying feedback reactions in a network based on dose-response titration and accurate quantification*
* *Aiming to get a better understanding of the feedback reactions that contribute to polarity*

1. **Spatial autofluorescence correction**

*Discussion of the problem of autofluorescence, and description of a method used to subtract autofluorescence from images on a pixel-by-pixel basis. Discussion of fluorophore bleedthrough. Possible comparison of two-channel vs three channel methods (eggshell correction).*

*Figures:*

* *Example N2 images, pixel-by-pixel regression*
* *Example of correction on a PAR-2 image*

1. **An accurate model for quantification of membrane and cytoplasmic protein**

*Previous models developed to quantify membrane and cytoplasmic concentrations have neglected out of focus light*

*Discussion of out of focus light in confocal images, possible solutions e.g. PSF prediction, bead imaging, z-stack imaging, and pros/cons of each*

*Description of a model developed to account for this out of focus contribution, giving more accurate quantification of membrane and cytoplasmic concentrations. The model must be calibrated, which can be done in two ways:*

* *Direct measurement of out of focus light using an eggshell stain*
* *Machine learning method (training on images of polarised cells)*

*Figures:*

* *A toy-model schematic demonstrating out of focus bleedthrough*
* *Optimised membrane and cytoplasmic reference profiles*
* *Example quantification of a PAR-2 image, extracted cytoplasmic and membrane components*
* *A comparison of training results from two different datasets (e.g. PAR-2 GFP vs Neon, PAR-2 wt vs C56S)*

1. **Validation of the quantification pipeline**

*Validation of the quantification pipeline with several control experiments, including PH rundown, N2 quantification, impact of pixel intensity noise*

*Figures:*

* *PH rundown: mem vs cyt quantification (including N2s), example images*
* *Mem and cyt quantification vs noise level for an example image*

1. **Conclusions**

* *We can successfully account for autofluorescence and out of focus light in order to accurately quantify membrane and cytoplasmic PAR concentrations from confocal images*
* *With this method, we can use dose-response assays to quantify the membrane binding kinetics of a protein, which can be compared to models to reveal underlying feedback reactions*

**4. QUANTITATIVE ANALYSIS OF PAR-2 KINETICS**

*Aims:*

* *Use a variety of techniques to quantify the molecular kinetics of PAR-2 in vivo*
* *Aiming to look for positive feedback, as a potential explanation for polarity maintenance in uniform aPAR systems, and because this has been suggested previously without quantitative evidence*
* *Comparison of wild type protein to RING mutant protein*

1. **PAR-2 displays non-linear membrane binding kinetics**

*Positive feedback models predict a nonlinear relationship between cytoplasmic and membrane concentrations, although the form of this relationship varies depending on the model*

*PAR-2 rundown and image quantification reveals non-linear membrane binding kinetics, suggesting positive feedback, however this relationship is not bistable*

*The relationship does not depend on the global presence of aPARs (polarised vs uniform vs S241A are all identical).*

*Figures:*

* *Mem vs cyt and dosage vs ratio quantification for PAR-2 in polarised and uniform conditions*
* *As above for uniform PAR-2 in par-3 mutant vs S241A conditions*
* *Example images, clearly showing a difference in affinity for different dosages*

1. **RING domain mutants display weaker nonlinearity**

*C56S mutant has weaker, more linear kinetics (not a simple rescaling of the wild type curve), indicating that positive feedback is affected*

*Additional RING domain mutants? E.g. second fold*

*Figures:*

* *Wild type vs C56S rundown data (mem vs cyt, dosage vs ratio)*
* *Example images*
* *Quantification of affinity and degree of non-linearity*

1. **RING domain mutation does not affect diffusion kinetics**

*Single particle tracking experiments show that wild type and C56S PAR-2 have near identical diffusion characteristics. In both cases there appears to be a mix of Brownian and subdiffusive particles, which we do not have an explanation for. Possibly compare regular Halo ligand with photoactivatable Halo ligand*

*Figures:*

* *Histogram of diffusion exponent for wt vs C56S*
* *Example tracks: Brownian vs subdiffusive*
* *Histogram of diffusion coefficient for wt vs C56S*

1. **Quantifying off rate kinetics**

*smPRESS results are difficult to interpret. Old FRAP data shows clear difference between wild type and C56S. Since diffusion is expected to be the same, this implies a large change in off rate. Could redo the experiment with CRISPR Neon lines, or reanalyse old data with constrained diffusion*

*Figures:*

* *TBD*

1. **Conclusions**

* *Quantitatively revealed a PAR-2 positive feedback reaction. Confirms previous suggestions for a positive feedback loop, although this is the first time this has been directly demonstrated*
* *Does not depend on PKC-3, but appears to depend on the RING domain (however it’s difficult to be sure that the RING is playing a direct role without more data).*
* *No clear difference in diffusion characteristics, although this cannot rule out a difference in membrane binding/oligomerisation that might be too small to detect*
* *Likely a difference in off rate, but need to confirm this*
* *Comparison to previous studies, especially Arata*
* *Notably, the positive feedback reaction is not sufficiently nonlinear to support bistability in polarity models, meaning that we cannot explain the ability of PAR-2 to polarise in uniform aPAR conditions. Nevertheless, it is worth exploring this reaction further to work out the molecular mechanism, and any potential effects that this has on the system.*

**5. DETERMINING MECHANISMS OF PAR-2 RING DOMAIN ACTION**

*Aims:*

* *Obtain direct evidence for the role of the RING domain in positive feedback*
* *Establish a molecular mechanism, exploring potential roles for ubiquitination and dimerisation*

1. **The RING domain is sufficient for cooperative membrane binding**

*A RING-PH fusion (lacking membrane localisation domain and phosphorylation sites of PAR-2) can communicate with endogenous full-length PAR-2 and polarise. The RING domain on its own does not bind to the membrane (from Hao et al., 2006, but need to replicate this with a CRISPR line).*

*Aim to do a rundown experiment with this protein to monitor the linearity of its kinetics, but need to generate a CRISPR mutant at the endogenous PAR-2 site, as my current mos site construct heavily silences*

*Figures:*

* *Images and quantification of RING::PH construct in wt, par-6 RNAi and par-2 RNAi backgrounds, and comparison to PAR-2 RING without PH (entirely cytoplasmic)*
* *Cyt vs mem relationship for RING::PH rundown*

1. **The RING domain is cis acting**

*RING mutants do not show altered behaviour in the presence of wild-type PAR-2. Suggests that the RING domain is cis-acting, which limits the number of potential models.*

*Figures:*

* *Bar chart showing PAR-2 membrane to cytoplasmic ratio for wt vs C56S vs C56S/wt*

1. **Exploring a potential role for autoubiquitination**

*Ubiquitylation assays do not show PAR-2 ubiquitylation activity (should confirm with an E2 scan). Unfortunately, negative results in an in vitro assay cannot rule out potential activity in all settings. Probe gel with anti-Ub antibody to confirm lack of any ubiquitination*

*(with Diego Esposito)*

*K94A (linchpin) mutant has identical membrane binding kinetics to wild type. Mutant is expected to disrupt ubiquitylation activity, whilst keeping the domain intact. However, given some uncertainty over the role of this site in other proteins, we cannot be certain that this is an effective mutant. Most RING domains with high sequence similarity have a K or R at this site, but some do not. Conserved in most (but not all) related species*

*Figures:*

* *Strep pull-down gel*
* *Ubiquitination assay gel*
* *PAR-2 RING sequence alignment vs PAR-2 in other worms and other RING domain proteins with high sequence similarity. Focussing on core RING domain*
* *Images and quantification of K94A vs wt*

1. **Exploring a potential role for RING domain dimerisation**

*Some prior work is consistent with PAR-2 being able to form homodimers (Arata, Motegi, Monica Gotta ongoing work), but functional roles and potential links to positive feedback has not been sufficiently explored.*

*Structure homology modelling predicts that the PAR-2 RING domain should be dimeric, via two alpha helices flanking the RING domain (setting up a 4 helix bundle). Mutations to cysteines in the RING domain prevent the domain from folding, which would presumably prevent this 4-helix bundle from forming.*

*Targeted mutation of sites at the predicted dimerisation interface would be predicted to resemble C56S phenotype (in progress)*

*In vitro assays. Planning to assess the dimerization activity of the PAR-2 RING domain (wt vs C56S) using in vitro biophysical assays. Initial contact made with Structural Biology STP, but work has not yet begun.*

*An in vivo dimerization assay (using the mitochondrial probe Tomm-20) does not show evidence for dimerization in vivo. However, it is unclear how sensitive this assay is, and it will likely fail to reveal weak/intermediate dimerization interactions. The result does, however, argue against constitutive dimerization (but need to validate that the method is effective using a constitutively dimeric positive control).*

*Figures:*

* *RING sequence alignments with structural information (focussed on flanking helices)*
* *Structure homology prediction*
* *Dimerisation site mutants: images and quantification*
* *Results from biophysical assays, to be determined*
* *Image of Tomm20::GBP::mKate, showing mitochondrial localisation*
* *Tomm20 pull-down images for PAR-2 GFP + mCherry, showing GFP localisation but not mCherry, and similar for positive control (e.g. PAR-6, PKC-3)*

1. **Conclusions**

* *PH::RING construct provides good direct evidence that the RING domain plays a role in cooperative membrane binding*
* *Given that the RING domain is cis acting, and linchpin mutant has no effect, ubiquitination seems an unlikely mechanism for regulating membrane affinity. PAR-2 may have some other role as a ubiquitin ligase, which could be context dependent (hence no positive result in in vitro assays), but this has not yet been explored.*
* *Pending some key results, but it seems likely that dimerization is the key difference between the wild type protein and RING mutants. It is likely that this is a weak/intermediate interaction (not constitutive). However, the link between dimerization and positive feedback has not been greatly explored, which is one aim of the next section*

**6. MODELLING THE IMPACTS OF PAR-2 DIMERISATION AND POSITIVE FEEDBACK**

*Aims:*

* *Fully characterise the link between protein dimerization and positive feedback, and the effects of dimer affinity*
* *Determine the potential impacts of positive feedback on polarity, using an extended form of the mutual antagonism model*

1. **Thermodynamic models of dimerization can recapitulate PAR-2 membrane binding kinetics** *(with David Zwicker)*

*Can get similar kinetics in models in which PAR-2 can only dimerise on the membrane vs ability to dimerise in membrane and cytoplasm.*

*Models give predictions for the relationship between dimerization strength and membrane-binding nonlinearity: monomers and dimers are predicted to have linear kinetics (higher affinity in the case of dimers), intermediate dimers are predicted to have non-linear kinetics (maximum predicted nonlinearity is quadratic).*

*Stronger non-linearities can be achieved by adding weak multivalent interactions.*

*A single model can be fit reasonably well to wild type and C56S rundown data, varying a single dimerization parameter*

*Figures:*

* *Fitting dimerization model to rundown data*
* *Figures demonstrating the general behaviour of the dimerization model, and the impact of dimerization strength*

1. **Positive feedback in mutual antagonism models**

*Assessing the implications of PAR-2 positive feedback in polarity models, with a focus on models that are constrained to reflect the limitations of a dimerization mechanism (i.e. detailed balance, max. quadratic nonlinearity).*

*Focussing on models constrained by PAR-2 rundown quantification, but possibly including a broader discussion of the effects of positive feedback and membrane affinity.*

*Figures:*

* *TBD*

1. **Conclusions**

* *Measured PAR-2 kinetics are compatible with a positive feedback loop driven by RING-dependent dimerisation*
* *Positive feedback from dimerization requires intermediate dimerization strength. Compatible with biophysical measurements?*
* *Positive feedback likely has some impact on the robustness and stability of polarity models, even when constrained by quantitative measurements, but I need to verify this*
* *NB. This is all in progress, and conclusions will be clarified as work progresses*

**7. EXPLORING THE IMPACTS OF PROTEIN DIMERISATION ON MEMBRANE BINDING**

*Aims:*

* *Establish a general link between protein dimerisation and membrane binding kinetics, particularly the importance of intermediate dimer strength, verifying predictions from thermodynamic models*
* *Work on this section is in very early stages, and progress may be limited by lack of time*

1. **Generating a constitutively dimeric form of PAR-2**

*PAR-2 with added GCN4 domain is predicted to be constitutively dimeric. Membrane affinity should be increased, but nonlinearity should be lost. C56S mutant is predicted to have no effect in these conditions.*

*May have implications for the system as a whole: look at viability etc.*

*If effective, validate dimerization through biophysical assays.*

1. **Restoring PAR-2 positive feedback through intermediate dimerization**

*GCN4 dimerization affinity can be altered through well characterised mutations. In theory, non-linear kinetics of C56S PAR-2 could be restored through the addition of an intermediate affinity GCN4.*

1. **Membrane binding rules in synthetic dimeric constructs**

*Aim to explore these concepts in entirely synthetic constructs (e.g. a dimerization domain bound to a membrane binding domain). Can we alter affinity and non-linearity by changing dimerization strength?*

1. **Conclusions**

* *Pending results*