The Institute of Cancer Research

PHD STUDENTSHIP PROJECT PROPOSAL

FUNDER DETAILS	
Studentship funded by:	Medical Research Council industrial Collaborative Awards in Science and Engineering (MRC iCASE)
Funder specific requirements:	All MRC iCASE students will attend taught courses one day a week for the first nine months of the PhD. This training will cover computational and thematic science training as well as core and transferable skills. Students will spend the remainder of the four years on their PhD project full time with monthly cohort activities. In addition, students must spend a cumulative period of no less than three months working in the facilities of the industrial collaborator. This 3 month period can be at any point during the studentship and may consist of a number of shorter visits if appropriate.
Estimated amount and distribution of time spent with industrial partner:	We expect that the student will spend 1 month with Intelligent Imaging Innovations Ltd during the first 6 months, for an introduction to the company and microscopy training. We expect that the student will spend a further 2 months with the company distributed in years 2 and year 3. The student will have the opportunity to spend time at the company's facility in Denver, Colorado.
PROJECT DETAILS	
Project Title:	**Using lattice light sheet microscopy to determine how the Spindle Assembly Checkpoint turns off**
SUPERVISORY TEAM	
Primary Supervisor:	Professor Jonathon Pines
Secondary Supervisor:	Dr Norman Davey
Industry Supervisor:	Benjamin Atkinson, Intelligent Imaging Innovations Ltd
Lead contact person for the project:	Jonathon Pines
DIVISIONAL AFFILIATION	
Primary Division:	Cancer Biology
Primary Team:	Cell Division

SHORT ABSTRACT

Unattached kinetochores prevent anaphase by generating an inhibitor composed of the MAD2, BUB3 and BUBR1 Spindle Assembly Checkpoint proteins. Kinetochores that stably attach to microtubules no longer generate the inhibitor but we do not understand how microtubule attachment does this. Is one attached microtubule sufficient, or are several required? Is the ability of microtubules to 'strip' kinetochores of the inhibitor proteins fast enough? To answer these questions we need to image microtubule attachment to kinetochores in real-time and quantify the changes in MAD2 and BUBR1 at kinetochores. We can now do this using lattice-lightsheet microscopy and endogenously tagged proteins.

BACKGROUND TO THE PROJECT

To maintain genomic stability the mechanisms of mitosis divide the genome such that the two daughter cells receive an equal and identical copy of the genome. To ensure this, the Spindle Assembly Checkpoint (SAC) prevents sister chromatid separation should any kinetochore fail to attach correctly to the spindle. We know that unattached kinetochores generate an inhibitor – the Mitotic Checkpoint Complex (MCC) - of the Anaphase Promoting Complex/Cyclosome (APC/C). The APC/C is an ubiquitin ligase that targets key mitotic regulators for ubiquitin-mediated proteolysis and thereby promotes sister chromatid separation and mitotic exit. The APC/C has to bind the Cdc20 protein to be active in mitosis, and Cdc20 is the target of the MCC. Our current understanding is that unattached kinetochores recruit components of the SAC, including the MAD1, MAD2, Bub1 and BubR1 proteins, and the Mps1 kinase, and these catalyse the formation of the MCC. Once a kinetochore attaches to spindle microtubules in a stable fashion, the kinetochore no longer catalyses MCC formation and this correlates with the loss of SAC proteins from the kinetochore (reviewed in Lara-Gonzales et al., 2021).

The SAC has the properties of being both potent – one unattached kinetochore can delay anaphase for several hours – but highly responsive: anaphase begins within a few minutes of the last kinetochore attaching to the spindle. The potency of the SAC is related to the ability of the MCC to inhibit two molecules of Cdc20, but our understanding of responsiveness is very rudimentary. In particular, we lack a mechanism for how microtubule attachment turns off the SAC. We know that microtubule attachment correlates with SAC protein loss from kinetochores, and that there is a dynein and Spindly-dependent mechanism to strip the MAD1/MAD2 heterodimer from kinetochores (Howell et al., 2001; Gassmann et al., 2010; Griffis et al., 2007)), but we also know that Spindly is not essential to turn off the SAC (Gassmann et al., 2010). Thus, it is likely that other mechanisms, such as post-translational modification of the SAC proteins and/or conformational changes in the kinetochore, are important to turn off kinetochore SAC signalling. This is the problem that we intend to address in this project

PROJECT AIMS

- Generate cells with endogenously tagged kinetochore and microtubule markers by CRISPR/Cas9 gene editing
- Optimise lattice light-sheet imaging to resolve kinetochore-microtubule attachment in living cells
- Measure the kinetics of MAD1, MAD2 and BUBR1 localisation to unattached kinetochores using existing endogenously tagged cell lines and lattice-lightsheet imaging and FRAP
- Determine the effect of microtubule attachment on MAD2 and BUBR1 residence at kinetochores
- Perturb known effectors of the microtubule attachment and the Spindle Assembly Checkpoint to determine how MAD2 and BUBR1 are removed from kinetochores upon microtubule attachment.

RESEARCH PROPOSAL

Experimental approaches:

We will use Retinal Pigment Epithelial cells (RPE1) as our experimental system. These are human cells that have a normal, stable karyotype and grow as a single cell sheet, which is required for high resolution microscopy. We have considerable experience in imaging mitosis in these cells and in editing their genes using CRISPR/Cas9 technology. We have already generated cell lines in which we have fluorescently-tagged the endogenous MAD1, MAD2 and BubR1 checkpoint proteins and the Mis12 kinetochore protein. We will use CRISPR/Cas9 to fluorescently-tag a microtubule end-binding protein (EB3), a protein that marks stable microtubule attachment (astrin or kinastrin) and a component of the RZZ-Spindly complex to monitor dynein-dependent stripping.

Once we have generated the cell lines, we will determine whether introducing the tag has perturbed the SAC or microtubule attachment using our live cell assays for SAC strength and chromosome alignment (Jackman et al. 2020). We have already validated tagged EB3 as a microtubule tracker and Mis12 as a kinetochore marker. Should the tag have perturbed the cells we will alter the position of the tag or choose another marker. We have plenty of experience in this after having to determine the correct internal site to tag MAD2.

With validated cell lines we can optimise imaging on the lattice-lightsheet microscope to resolve microtubule attachment to kinetochores. Tackling this has been difficult because of limitation in live-cell imaging. Kinetochores are smaller than the normal resolution of the light microscope, and mitotic cells are highly sensitive to light. This causes problems in visualising microtubule attachment, which is a very dynamic process and thus needs high temporal and spatial resolution imaging. The advent of the lattice-lightsheet microscope (Chen et al., 2014), which can image at high speeds but with very little photo-damage means that we can now approach the problem. We will almost certainly need to use structured illumination (SIM) to resolve the attachments of single microtubules and collaborating with Intelligent Imaging on this will be crucial for success. Once we have optimised this assay, we will analyse the behaviour of the SAC proteins, particularly MAD1 and MAD2, to determine how the population of MAD1 and MAD2 at individual kinetochores responds to microtubule attachment. We will be particularly interested to determine whether MAD1 and MAD2 have the same behaviour in response to attachment because this will reveal whether the control is primarily on MAD1, whose localisation depends on the RZZ complex, or whether MAD2 post-translational modification may also be involved. We will use Fluorescence Recovery After Photobleaching (FRAP) to measure the flux of MAD2 at unattached kinetochores and how this changes upon attachment. We will also introduce dynein inhibitors (Ciliobrevin/Dynapyrazole/Dynarrestin) to prevent MAD1 and MAD2 ‘stripping’ and determine the effect on their dynamical behaviour in the presence and absence of the Spindly protein.

These experiments will form the basis for hypotheses to explain how microtubule attachment turns off the SAC. Our quantitative experiments will determine whether there is a simple inverse relationship between number of microtubules attached and number of SAC proteins at a kinetochore (unlikely) or whether SAC proteins exhibit switch-like behaviour, as previously reported for exogenous markers (Kuhn and Dumont, 2019). If SAC proteins do have a switch-like response we will determine whether this is controlled by Spindly and dynein-stripping of MAD1 and/or whether post-translational modifications of MAD1 and MAD2 are involved by introducing mutants of known phosphorylation sites.

At the end of the project we should have a much better understanding of kinetochores integrate microtubule attachment state with the SAC.

Benefit of collaborating with Intelligent Imaging Innovations:

Intelligent Imaging is constantly developing the lattice-lightsheet microscope to enhance its capabilities for quantitative imaging. Most recently, Intelligent Imaging have introduced a photobleaching laser to enable the measurement of flux through a specific structure in a cell. We wish to take advantage of this to develop the ability to measure flux through kinetochores in a mitotic cell. This will be challenging as kinetochores are sub-resolution structures that move and mitotic cells are highly light-sensitive. Collaborating with Intelligent Imaging will enable us to take advantage of their expertise in optimising the optics and image processing of lattice-lightsheet microscopy.

LITERATURE REFERENCES

Chen, B., et al., Lattice light-sheet microscopy: Imaging molecules to embryos at high spatiotemporal resolution, Science, (2014), DOI: 10.1126/science.1257998

Gassmann, R., Holland, A.J., Varma, D., ,Wan, X., ,Civril, F., Cleveland, D.W, Oegema, K., Salmon, E.D, Desai, A., Removal of Spindly from microtubule-attached kinetochores controls spindle checkpoint silencing in human cells Genes Dev., http://www.genesdev.org/cgi/doi/10.1101/gad.1886810. (2010).

Eric R. Griffis,E.R., Stuurman,N. and Vale, R.D, Spindly, a novel protein essential for silencing the spindle assembly checkpoint, recruits dynein to the kinetochore *J Cell Biol.* (2007) https://doi.org/10.1083/jcb.200702062

B.J. Howell, B.F. McEwen, J.C. Canman, D.B. Hoffman, E.M. Farrar, C.L. Rieder, E.D. Salmon, Cytoplasmic dynein/dynactin drives kinetochore protein transport to the spindle poles and has a role in mitotic spindle checkpoint inactivation J. Cell Biol. (2001) https://doi.org/10.1083/jcb.200105093

Jackman, MR, Marcozzi, C., Barbiero, M., Pardo, M., Yu, L., Tyson, A.L., Choudhary, JS., Pines, J. (2020) Cyclin B1-Cdk1 facilitates MAD1 release from the nuclear pore to ensure a robust spindle checkpoint *J. Cell Biol*., doi.org/10.1083/jcb.201907082

Kuhn, J and Dumont, S., Mammalian kinetochores count attached microtubules in a sensitive and switch-like manner, J. Cell Biol., (2019) https://doi.org/10.1083/jcb.201902105

Lara-Gonzales, P., Pines, J., and Desai, A., Spindle assembly checkpoint activation and silencing at kinetochores, Seminars in Cell and Developmental Biology, (2021) https://doi.org/10.1016/j.semcdb.2021.06.009

CANDIDATE PROFILE

Note: the ICR's standard minimum entry requirement is a relevant undergraduate Honours degree (First or 2:1)

Pre-requisite qualifications of applicants:	BSc in biological science or in optics-related discipline
Intended learning outcomes:	High resolution light microscopy Quantitative image analysis CRISPR/Cas9 gene editing

ADVERTISING DETAILS	
Project suitable for a student with a background in:	☒ Biological Sciences ☒ Physics or Engineering ☐ Chemistry ☐ Maths, Statistics or Epidemiology ☐ Computer Science ☐ Other (provide details)
Keywords:	1. Imaging
	2. Mitosis
	3. Genome stability
	4. Kinetochore
	5. Super-resolution
	6.