Molecular and Genetic analysis of *Drosophila Rad21*: A gene and protein involved in sister chromatid cohesion

Thesis submitted by
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in October 2005

for the degree of Doctor of Philosophy
in the School of Pharmacy and Molecular Sciences
James Cook University

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STATEMENT OF SOURCES

DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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STATEMENT OF THE CONTRIBUTION OF OTHERS

Stipend support: I was a recipient of an Australian Postgraduate Award stipend, and also of a three month School of Pharmacy and Molecular Sciences stipend.

Supervision: This research was performed under the supervision of Dr W. Warren in the School of Pharmacy and Molecular Sciences, at James Cook University, Townsville.

Other collaborations: Part of this work involved a four week period in the laboratory of collaborator, Dr. Margarete Heck in Edinburgh during which general techniques in biochemistry were learned.

Editorial assistance: Drs. W. Warren and K. Gorringe kindly proof-read this thesis

Research assistance: General technical and research assistance was provided by Leigh Cuttell. Additional technical assistance was provided by numerous individuals over the three year period of this study.

DNA microinjections were performed by Dr. W Warren.

Samples to be analysed by scanning electron microscopy were sputter coated by Dr. Kevin Blake.

Any other assistance: Analytical assistance in the form of DNA sequence analysis and microscopy was provided by the Advanced Analytical Centre at James Cook University.

Project costs: This project was supported in part by research grants from the Australian Research Council and The March of Dimes Birth Defects Foundation

ACKNOWLEDGEMENTS

There are so many people that have helped me over the last few years both scientifically and away from work.

I would firstly like to thank my supervisor, Dr Bill Warren, for his support throughout this project. I am grateful for the many opportunities that you have offered me, including moving interstate, and setting up a new lab. I am especially grateful that you had enough trust in me and my scientific ability to hand over your pet project and let me run with it. Just having that freedom has helped me grow immensely as a scientist, thank you.

To my collaborators and co-workers, thanks for all of the inspiration and distractions. Margarete, thank you for welcoming me into your lab in Edinburgh, and Sharron thanks for giving me a crash course in *Drosophila* biochemistry. Without your selflessness in helping me I am sure that it would have taken me eons to be able to generate pretty blots! Big thanks goes to the succession of technical assistants that have kept me supplied with fly and bacteria media over the years, without your help the lab would not have run so smoothly. To Leigh, Rachael, Sally and Kylie, thank you for everything. Without you guys, counting flies would never have been so much fun. Thanks especially Kylie and Rach for all of the scientific discussions and brain storming in the office, and for listening to me complain when things weren't working.

Dr. Kevin Blake, thank you for teaching me how to generate beautiful scanning electron micrographs, and for sputter coating the 300 plus samples that I continued to bombard you with. Thanks for the laughs and the genuine interest in my work. To Dr. Helena Richardson and Michelle Coombe, thank you for sending me part of the deficiency kit so that I could commence the genome-wide screen. Leonie, Hannah and Nicole, thank you for answering my calls for help when I was frustrated over technical problems, collectively you saved me hours of frustration and hair pulling. Bill, thanks for performing the embryo microinjections.

To all of my friends that have continued to support me despite the huge distance between Townsville and Melbourne, thank you. I wish to thank my family who have supported me in everything that I do. Mum, Dad, Paul, Amie, Sarah, Rach and Tom thanks for knowing that I am always there for you and care even if I am physically miles away.

Last but never least, I could not have done any of this without Wayne. Thank you for being my rock and my point of sanity throughout all of this.

It certainly has been an experience, thank you to everyone that has shared it with me.

ABSTRACT

The accurate and efficient dissemination of replicated chromosomes into daughter cells is fundamental to all aspects of biology. Chromosomal missegregation can lead to aneuploid chromosome configurations which are a hallmark of cancer cells and also a leading cause of birth defects and infertility in humans. Given that chromosome missegregation can result in such disastrous consequences, cells have evolved mechanisms to ensure the faithful segregation of chromosomes, one of which is sister-chromatid cohesion which is mediated by the cohesin complex.

Cohesin is a multi-protein complex thought to be the primary effector of sister chromatid cohesion in all eukaryotes. In yeast, cohesin is loaded onto chromosome arms in S-phase where it maintains sister chromatid cohesion until the metaphase-anaphase transition. Sister chromatid separation is then triggered by the site-specific cleavage of the RAD21 cohesin subunit. In metazoan species, including *Drosophila*, the bulk of cohesin dissociates form chromosomes in prophase, leaving a minor pool of centromere-associated cohesin to maintain sister-chromatid cohesion until anaphase. Exactly how the various cohesin subunits and their regulators orchestrate these events has yet to be fully elucidated. Meiotic cohesin complexes are subjected to additional levels of regulation to accommodate the different types of cell division that occur to produce haploid gametes. In humans, premature loss of meiotic sister chromatid cohesion has been proposed as the most likely molecular cause for sporadic aneuploidy linked to advanced maternal age.

The results presented in this thesis begin with a description of the rationale and approach used to identify the DRAD21 separase cleavage sites, and subsequently mutate them using site-directed mutagenesis. Characterisation of the dominant alleles generated is described, as is the first evidence of DRAD21 proteolysis. The overexpression of non-cleavable DRAD21 isoforms was investigated in a range of different tissues and developmental stages, and was shown to dominantly reduce the size of adult tissue. These data suggested that overexpression of non-cleavable isoforms of DRAD21 in dividing cells increased levels of cell death. Analysis of the cellular effects of non-cleavable DRAD21 overexpression in the developing eye imaginal disc confirmed that the level of apoptosis was increased in cells expressing non-cleavable DRAD21, and that this DRAD21 isoform induced mitotic delay or

arrest, consistent with a defect in mitotic progression. This is the first description of a *Drad21* mutant phenotype. The reduced and roughened eye phenotype generated as a result of non-cleavable DRAD21 expression in the eye imaginal discs provided a tool to use in genetic studies of DRAD21 function.

Genetic analysis showed that known and predicted cohesin regulators are capable of modulating the DRAD21 eye phenotype, therefore establishing the suitability of this phenotype for use in a genetic screen. The entire *Drosophila* genome was screened for genetic modifiers of the DRAD21 eye phenotype. In total 62 interacting genomic regions were identified, spanning chromosomes two, three and four. Analysis of these interactions revealed both enhancers and suppressors of the DRAD21 eye phenotype, and genetic dissection of some of the interacting regions allowed 13 modifier loci to be unequivocally identified at the molecular level. Specifically, ten distinct interacting regions were examined at the molecular level and ten suppressor loci and three enhancer loci were identified and a mechanism by which these interactions may be occurring was proposed for each.

These studies are likely to significantly influence our current understanding of metazoan chromosome dynamics and identify novel regulators of chromosome segregation. To date, some interacting loci identified at the molecular level have established roles in chromosome cohesion, while for others this study provides the first evidence for their role in this process. These studies will identify both novel regulators of chromosome segregation and hopefully provide a shortlist of genes that when functionally impaired may incrementally increase the risk of chromosome missegregation and aneuploidy in humans.

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LIST OF ABBREVIATIONS

aa	Amino Acid
BL	US *Drosophila* stock centre at Bloomington, Indiana
bp	Base pair
BSA	Bovine serum albumin
cDNA	DNA that is synthesised from a messenger RNA template
CO_2	Carbon dioxide
Df	Deficiency
dH_2O	Distilled water
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
ECL	Enhanced chemi-luminescence
EDTA	Ethylenediaminetetraacetic acid
G0	Parental generation
G1	First generation
G2	Second generation
GMR>	GMR-Gal4 induced transgene expression
HPLC	High Performance liquid chromatography
IPTG	Isopropyl-beta-D-thiogalactopyranoside
kb	Kilobase
kDa	Kilodalton
LB	Luria Bertani media
MF	Morphogenetic furrow
PBS	Phosphate buffered saline
PBT	Phosphate buffered saline plus 0.1% Tween-20
PCR	Polymerase chain reaction
rpm	Revolutions per minute
SAP	Shrimp Alkaline Phosphatase
SB	Squishing buffer
SDS	Sodium dodecylsulfate
SMC	Structural maintenance of chromosomes
UAS	Upstream activation sequence
X-Gal	5-bromo-4-chloro-3-indolyl-bD-galactoside

Addendum and Erratum

The following information should be added to, and/or considered with, the indicated thesis sections.

Chapter 1: Introduction

Figures 1.2 (on page 6) and 1.3 (on page 7) insert the following after the first sentence of each figure legend:

The centrosome, indicated as a black dot, is the microtubule organising centre of animal cells and also replicates during cell division to form the spindle poles.

Figure 1.5 on page 16; insert the following after the third sentence of the figure legend:

Posterior is to the left and anterior to the right.

Chapter 2: Materials and Methods

Section 2.6.5: Screening for genetic interactors. On page 55, insert the following after the first complete sentence on this page:

When comparing the effect of halving the dose of one or a number of genes by comparing affected and control siblings it was necessary to compare flies of the same sex. This is necessary as the male flies are smaller than the females, and therefore have smaller eyes.

Chapter 3: Generation and analysis of a dominant *Drad21* allele

Sections 3.2.4.3 and 3.2.5.2: On pages 77 (section 3.2.4.3) and 82 (section 3.2.5.2) the following needs to be added after the final sentence of each section:

The observed and/or predicted apoptosis may not be directly due to the expression of $Drad21^{DM}$, but a secondary effect due the inability to efficiently segregate chromosomes at anaphase. Irrespective of the direct apoptotic trigger, it is indisputable that the dominant phenotypes observed are at least in part due to an increase in the level of apoptosis.

Table 3.2 on page 67, the following should replace the descriptor "none" in the table:

WT

Figures 3.9 (page 76) and 3.10 (page 78), insert the following after the final sentence of each figure legend:

Anterior is to the left and posterior to the right.

Figure 3.11 on page 80, insert the following before the final sentence of the figure legend:

Overexpression of cyclinE using the GMR driver induces additional S-phases and subsequent mitoses which ultimately results in the differentiation of extra-numerary ommatidia and an enlarged, roughened and disorganised eye phenotype (see also sections 1.2.2.1 and 1.2.2.2).

Section 3.2.5.1: Ectopic expression of cleavage-resistant DRAD21 isoforms in the asynchronously cycling cells of the eye imaginal disc. On page 81, replace paragraph two, sentence four with:

In contrast, overexpression of one copy of the $Drad21^{RA474AG}$ or of $Drad21^{DM}$ transgenes in this pattern resulted in a reduction in the amount of adult eye tissue but did not appear to affect the organisation of the ommatidia to anywhere near the same extent as GMR driven expression.

Section 3.2.2: A role for DRAD21 in apoptosis? On page 85, replace paragraph one, sentence three with:

Cleavage of human RAD21 by caspases 3 and 7 has been shown to occur before the condensation of apoptotic chromatin and the resultant ~65kDa protein is required to amplify the cell death signal within the cell (Chen et al.,2002, Pati et al., 2002). Whether caspases are capable of cleaving *Drosophila* RAD21 could be assayed using commercially available enzymes and immuno-purified DRAD21 as a substrate. Additional investigations into the role of DRAD21 in apoptosis could also involve *in silico* analyses of the protein sequence to identify likely caspase cleavage sites and overexpression of the predicted cleavage products in flies and cell culture to see if they were capable of inducing apoptosis.

Chapter 4: Genome-wide modifier screen to identify novel regulators of metazoan chromosome segregation

Table 4.1 on page 92; insert the following columns after column titled "Gene Name":

Function	Page reference
Cohesin	22-24, 27-28, 30, 35-36, 137, 173 and 183
DNA replication (PCNA)	160
Proteolysis	116
DNA helicase	
Chromosome segregation	27-33, 35-36, 43, 57-59, 61, 63, 70, 72-75, 84, 90-92, 114-115, 176-177, 181-183, 189-90
Mitotic kinase	29
DNA damage checkpoint	
Proteolysis	116
Mitotic kinase	181 and 183
separase	27-33, 35-36, 43, 57-59, 61, 63, 70, 72-75, 84, 90-92, 114-115, 176-177, 181-183, 189-90
DNA helicase	
Mitotic checkpoint	
DNA damage response	
Proteasome subunit	
Proteolysis	116
Proteolysis	
Mitotic exit	
Condensation and cell proliferation	147-148, 150-151, 180-181
Cell cycle progression	
Sister chromatid cohesion	
Mitotic kinase	27, 29, 163, 181, 183
Mitotic kinase	27, 29, 163, 181, 183
Proteasome	
securin	27,32-33,35,126-127, 176
Mitotic progression, anaphase entry	126-127, 154-155, 190
development	
Transcription, cohesion loading	21, 24-25, 35, 90-91,114-115, 140-141, 183 and 194
coheisn	22, 24,27-28, 30, 35-36, 137, 173 and 183
Centromeric cohesion	31, 35 and 114

Chapter 5: Identification of genetic modifier loci at the molecular level

Section 5.2.2.1: S122 Region overview. On page 123 replace paragraph one sentence two with:

> This phenotype is characterised by a weak suppression of both the size and organisation defects of the GMR>DRAD21^{DM} eye phenotype (Figure 4.9, Table 4.5).

Section 5.2.2.2: Genetic dissection of region S122. On page 124 replace paragraph two sentence two with:

> Both *CycA* alleles tested were capable of suppressing the reduced and roughened GMR>DRAD21DM eye phenotype to similar extents (Figure 5.2).

Section 5.2.3.1: S107 Region overview. On page 128 insert the following after the final sentence of paragraph two:

> PTEN is an important cell growth regulator and plays an essential role in the regulation of both cell growth and cell division (Gao et al., 2000) through suppression of PI3K-dependent signaling (Maehama et al., 2004).

Section 5.2.5.2: Genetic dissection of region S67. On pages 140-141, replace the sentence that spans these pages with the following:

> All four were shown to modulate the GMR>DRAD21DM phenotype, primarily by increasing the organisation of the eye (Figure 5.10).

Section 5.2.6.2: Genetic dissection of region S81. On page 145 replace paragraph two with the following:

> LILLIPUTIAN is a protein involved in the regulation of cell size and cytokinesis (Maehama et al 2004, Tepass et al 2001) and is encoded by *lilli* located at 23B7-23C2. *lilli* genetically interacts with the *Pten* tumour suppressor gene (Maehama et al 2004), further supporting its role in the correct execution of the cell cycle, however, the *lillik*^{05431} insertion allele failed to significantly modify the GMR>DRAD21DM phenotype.

Section 5.2.10.2: Genetic dissection of region E60. On page 164 replace the final sentence of the last paragraph with the following:

> *Src42A*^{KG02515} suppresses both the size and organisation defects to a moderate extent, whilst *Scr42A*^{myri}and *Src42A*^{E1} mildly suppress the size and organization defects. This difference in the level of suppression is most likely to be due to the different strengths of the alleles tested, with the *Src42A*^{KG02515} insertion allele retaining the least amount of wild-type function.

5.2.10.3.3 Proposed mechanism of suppression by Src42A. On page 123 replace the fourth sentence of this section with the following:

> *Drosophila* C-terminal SRC kinase, CSK, negatively regulates SRC42A mediated signaling, and SRC42a has been demonstrated to signal through JNK and JAK/STAT pathways to promote proliferation, and through the JAK/STAT pathway to trigger apoptosis (Read et al 2004, Laberge et al., 2005).

References cited in Addendum that do not appear in bibliography

MAEHAMA, T., KOSAKA, N., OKAHARA, F., TAKEUCHI, K.-I., UMEDA, M., DIXON, J. E. & KANAHO, Y. (2004) Suppression of a phosphatidylinositol 3-kinase signal by a specific spliced variant of Drosophila PTEN. *FEBS Letters,* 565**,** 43-47.