Investigation of pathways responsible for repeat RNA-mediated cellular perturbation in *Drosophila* models of dominant expanded repeat disease

A thesis submitted for the degree of Doctor of Philosophy
September 2011

Kynan Thomas Lawlor, B.Sc. (Mol. Biol.) (Hons.)

Discipline of Genetics
School of Molecular and Biomedical Science
The University of Adelaide
# Table of Contents

Table of Contents ........................................................................................................ iii  
Index of Figures and Tables .................................................................................. vi  
Declaration .............................................................................................................. ix  
Acknowledgements ................................................................................................ xi  
Abbreviations .......................................................................................................... xii  
Abstract .................................................................................................................... xv  

## CHAPTER 1: Introduction and background ..................................................... 1  

1.1 Human expanded repeat disease ........................................................................... 1  
   1.1.1 A common molecular basis for dominant expanded repeat disease ................. 1  

1.2 Expanded polyglutamine protein repeat-mediated pathology ......................... 5  

1.3 Expanded repeat RNA-mediated pathology ......................................................... 7  
   1.3.1 Myotonic dystrophy type 1 (DM1) and 2 (DM2) ................................................ 7  
   1.3.2 Fragile X tremor ataxia syndrome (FXTAS) ..................................................... 9  
   1.3.3 Spinocerebellar ataxia type 8 (SCA8) ............................................................... 11  
   1.3.4 Huntington’s disease like-2 (HDL-2) ............................................................... 12  
   1.3.5 Spinocerebellar ataxia type 10 (SCA10) ......................................................... 12  
   1.3.6 Spinocerebellar ataxia type 12 (SCA12) ......................................................... 13  
   1.3.7 RNA-mediated pathology in the polyglutamine diseases .............................. 13  

1.4 Pathways of repeat RNA-mediated pathology ............................................... 15  
   1.4.1 Hairpin-forming RNA as a pathogenic agent .................................................. 15  
   1.4.2 Sequestration of MBNL-1 and other proteins ............................................... 17  
   1.4.3 Small RNA processing pathways and bi-directional transcription ............... 20  

1.5 Repeat RNA as a common contributor to dominant expanded repeat disease ................................................... 23  

1.6 Drosophila as a model for dominant expanded repeat disease ....................... 26  
   1.6.1 A Drosophila model to examine pathways of repeat RNA-mediated pathology ................................................... 27  

## CHAPTER 2: Materials and Methods .............................................................. 31  

2.1 Materials ........................................................................................................... 31  

2.2 Methods .......................................................................................................... 35
Summary of results........................................................................................................... 41

CHAPTER 3 : Specific cellular perturbation due to ubiquitous expression of expanded repeat RNA in Drosophila.................... 43

  3.1 Ubiquitous expression of CUG or CAG repeat RNA causes reduced viability in Drosophila ........................................................................................................... 45
  3.2 Ubiquitous expression of CUG or CAG repeat RNA causes disruption to adult Drosophila tergite patterning ..........................49
  3.3 Repeat RNA expression in developing histoblast cells is sufficient to cause tergite disruption.................................................................55
  3.4 Examining the effect of reduced muscleblind levels on RNA-mediated tergite disruption ................................................................. 58
  3.5 Chapter discussion ............................................................................................. 63

CHAPTER 4 : Repeat RNA nuclear localisation in Drosophila ....... 67

  4.1 CUG repeat RNA forms specific nuclear foci within Drosophila larval muscles ................................................................................................. 68
  4.2 CAG repeat RNA does not form muscle-specific nuclear foci..................72
  4.3 Non hairpin-forming CAA repeat RNA shows similar localisation to CAG repeat RNA ................................................. 74
  4.4 Repeat sequence specific localisation patterns are independent of transcript context .............................................................. 76
  4.5 Repeat RNA foci are not observed in adult Drosophila brains .......... 80
  4.6 Chapter discussion ............................................................................................. 82

CHAPTER 5 : Characterisation of dominant phenotypes from expression of a specific \( rCAG_{-100} \) transgene insertion................. 85

  5.1 Expression of \( rCAG_{-100} [\text{line C}] \) is sufficient to cause dominant phenotypes in Drosophila ........................................................................................................... 86
  5.2 \( rCAG_{-100} [\text{line C}] \) is inserted at the cheerio locus ........................................... 90
  5.3 \( rCAG_{-100} [\text{line C}] \) enables bi-directional expression of an expanded repeat 94
  5.4 Ectopic expression of \( rCAG_{-100} [\text{line C}] \) leads to loss of photoreceptors .....99
  5.5 Ubiquitous expression of \( rCAG_{-100} [\text{line C}] \) leads to reduced lifespan...... 103
  5.6 Pan-neuronal expression of \( rCAG_{-100} [\text{line C}] \) leads to neuronal defects.. 105
CHAPTER 6: Comparison of pathways responsible for double-stranded and hairpin-forming repeat RNA-mediated pathology... 111

6.1 Comparison of neuronal bi-directional repeat expression from rCAG$_{100}$ [line C], and complementary repeat expression from different loci........... 113
6.2 Altering Dicer-2 levels does not significantly alter rCAG$_{100}$ [line C] photoreceptor degeneration................................................................. 116
6.3 Examining the role of Dicer processing pathways in hairpin RNA-mediated tergite phenotypes ................................................................. 119
   6.3.1 Dicer-2 modification of tergite phenotypes. ................................. 120
   6.3.2 Dicer-1 modification of tergite phenotypes. ................................. 124
6.4 Chapter discussion ............................................................................. 128

CHAPTER 7: Final discussion ................................................................. 131

7.1 Summary of results ........................................................................... 131
7.2 Pathways of hairpin RNA-mediated pathology.................................. 133
7.3 Double-stranded repeat RNA-mediated pathogenesis...................... 135
7.4 Multiple pathways contribute to expanded repeat disease............... 136
7.5 Future directions ................................................................................ 140

Appendices............................................................................................. 143

Appendix 1 .............................................................................................. 143
Appendix 2.1 ........................................................................................... 144
Appendix 2.2 ........................................................................................... 145
Appendix 3.1 ........................................................................................... 146
Appendix 3.2 ........................................................................................... 147
Appendix 3.3 ........................................................................................... 148
Appendix 3.4 ........................................................................................... 149
Appendix 4 .............................................................................................. 151

References ............................................................................................. 173
Corrections

Chapter 1
Page 10, paragraph 2 should read “rather than enhancement”

Chapter 2
Page 35, Quantification of tergite disruption, should include the paragraph:
The scoring scheme was based on the number and severity of disrupted tergites, using particular morphological attributes to define each category, thus minimising any experimenter bias. Preliminary data showed no significant difference (data not shown) between populations when scoring ‘experimenter blind’. As such, remaining experiments were not scored blind. The order in which genotypes were scored each day was randomised and data from multiple sets of progeny obtained from multiple sets of parents on different days was used in each case.

Page 36, Quantification of locomotion phenotype, should include the paragraph:
Scoring involved reviewing the video to tally the time in seconds that each fly spent either upright (walking or standing) or on its back. As the possibility for experimenter bias in this case appeared negligible scoring was not done ‘blind’.

Page 40, Climbing assays, should include the clarification:
\( n = 3 \) biological replicates (sets), with 20-25 animals per genotype, per biological replicate (set), for a total of 60-75 animals examined for each genotype. A climbing score representing each biological replicate (set) was obtained by calculating the mean from 5 consecutive trials for each genotype. A final genotype score was obtained by calculating the mean of all 3 biological replicates.

Chapter 3
Page 46, Figure 3.1 legend should include the paragraph:
Fisher’s exact test does not include a calculation of standard deviation, or standard error, however 95% confidence intervals were calculated for each particular proportion. As this involved a separate calculation these values are included in Appendix 1, rather than as error bars.

Chapter 4
Page 69, In Figure 4.1 C, DAPI staining was poorly reproduced in the printed version. Images were chosen based on being representative of each genotype in regard to repeat RNA staining (Cy3 signal), with DAPI included as a guide to the location of the nucleus only. As such the relative levels of DAPI signal do not change the interpretation of the data. A modified version (to improve visibility in printed form) of the DAPI staining shown in 4.1 C is included below.

![Image of DAPI staining with scale bar]
In this study CUG-specific RNA localisation patterns were observed in independent samples from independent transgenic lines and thus the result appears robust. However, as quantification of foci was not performed, further analysis would be necessary to confirm the more subtle differences in CUG-specific localisation patterns observed in different repeat expression contexts.

Confocal microscopy was not performed in this case. Techniques allowing higher imaging resolution may confirm the absence of neuronal foci in *Drosophila* with more certainty.

Scale bars were initially not included in fluorescent micrographs. Examples for each type of image taken are included below to aid in interpretation of these results.

An example of muscle nuclei (as in 4.1, 4.3, 4.4, 4.5). All images were captured and cropped in the same way such that scale is identical:

An example of an adult brain at higher magnification (as in 4.6 B-D):
An example of an adult brain at lower magnification (as in 4.6 A). In this case landmarks within the brain are annotated to further aid in interpretation.

Page 82, paragraph 1, should read:
“... support the conclusion that pathways …..”

Chapter 5
Page 89, paragraph 3, should read:
“... indicate that rather than the insertion directly disrupting ….”

Chapter 6
Page 118, Figure 6.2 figure legend, should state:
All flies were aged for 35 days before sectioning (Materials and Methods).

Chapter 7
Page 135, paragraph 2, should read:
“In support of this we see alterations to miRNA profiles....”

Page 135, paragraph 3, should read:
“... indicating that, as in our model, complementary transcripts form double-stranded RNA that is processed.”
### Index of Figures and Tables

Table 1.1 Common features of the human dominant expanded repeat diseases ..........2  
Figure 1.1 Gene location of repeat tracts causing dominant expanded repeat disease ..4  
Figure 1.2 Hairpin-forming repeat RNA-mediated sequestration ..............................16  
Table 1.2 Evidence for pathways involving sequestration of MBNL-1 as a common contributor to pathology. .................................................................18  
Figure 1.3 Bi-directional transcription of repeat-containing genes ..........................21  
Figure 1.4 Proposed pathways of RNA-mediated pathology ...............................25  
Figure 1.5 A system to examine repeat RNA pathology in Drosophila ....................29  
Figure 3.1 Ubiquitous expression of hairpin forming repeat RNA leads to reduced viability.................................................................46  
Figure 3.2 Tergite disruption is observed in rCUG-100 and rCAG-100 expressing flies .................................................................50  
Figure 3.3 Comparison of tergite disruption in independent repeat lines and controls. .................................................................52  
Figure 3.4 Expression of rCAG-100 in histoblast cells leads to mild tergite disruption .............................................................................57  
Figure 3.5 Effect of reducing muscleblind levels on tergite disruption ..................60  
Figure 3.6 Statistical analysis of phenotypic changes due to reduced muscleblind levels .............................................................................62  
Figure 4.1 Localisation of rCUG-100 in Drosophila muscle nuclei .......................69  
Figure 4.2 Muscle specific rCUG-100 localisation ............................................71  
Figure 4.3 Localisation of rCAG-100 in Drosophila muscle nuclei .......................73  
Figure 4.4 Localisation of rCAA-100 in Drosophila muscle nuclei .......................75  
Figure 4.5 Nuclear localisation of repeats expressed within a GFP transcript........78  
Figure 4.6 Nuclear foci are not detected in adult Drosophila brains ..................81  
Figure 5.1 Ectopic expression of rCAG-100 [line C] is sufficient to cause locomotion defects and disruption to the patterning of the eye ........................................87  
Figure 5.2 The rCAG-100 [line C] insertion is within the cheerio gene ...............91  
Figure 5.3 rCAG-100 [line C] insertion phenotypes are not caused by a decrease in cheerio levels .............................................................................93  
Figure 5.4 The rCAG-100 [line C] insertion is transcribed to produce a complementary rCUG repeat transcript .........................................................96
Figure 5.5 Ectopic expression of rCAG-100 [line C] in the eye leads to photoreceptor degeneration................................................................. 100
Figure 5.6 Ubiquitous expression of rCAG-100 [line C] leads to a reduction in lifespan. ................................................................. 104
Figure 5.7 Pan-neuronal expression of rCAG-100 [line C] leads to a reduction in climbing ability. ................................................................. 106
Figure 6.1 Comparison of bi-directional and complementary repeat expression in neurons. ................................................................. 115
Figure 6.2 Increased Dcr-2 levels does not significantly modify rCAG-100 [line C] photoreceptor degeneration ................................................................. 118
Figure 6.3 Population distribution of tergite phenotype severity with reduced Dicer-2 levels ................................................................. 121
Figure 6.4 Analysis of the effect of reducing Dicer-2 levels on the total phenotype proportion and proportion with a strong phenotype........................................ 122
Figure 6.5 Population distribution of tergite phenotype severity with reduced Dicer-1 levels ................................................................. 125
Figure 6.6 Analysis of the effect of reducing Dicer-1 levels on the total phenotype proportion and proportion with a strong phenotype................................. 127
Figure 7.1 Multiple pathways leading to cellular perturbation in Drosophila models of expanded repeat disease. ................................................................. 139
Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Kynan Lawlor and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University’s digital research repository, the Library catalogue, the Australasian Digital Thesis Program (ADTP) and the also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Kynan Thomas Lawlor
Acknowledgements

I wish to thank the following for their contribution to this project: my supervisor Robert Richards for wise guidance and support; Louise O’Keefe, my second supervisor, for excellent ideas, mentorship and support, especially during the writing of this thesis. Past and present members of the Richards lab for expert assistance with experiments and sharing their wisdom and good humour over the years, especially Clare van Eyk, Saumya Samaraweera, Amanda Choo and Sonia Dayan; my friends and family, particularly Merridy Lawlor for always being there and finally, my wife Jessica, as this work would not have been possible without her endless encouragement, support and patience.
Abbreviations

°C : degrees Celsius
% : percentage
µg : micrograms
µL : microlitre
µm : micrometre
A : adenosine
AR : androgen receptor
ATN1 : atrophin 1
ATXN1 : ataxin 1
ATXN2 : ataxin 2
ATXN3 : ataxin 3
ATXN7 : ataxin 7
ATXN8OS : ataxin 8 opposite strand
ATXN10 : ataxin 10
BAC : bacterial artificial chromosome
bp : base pairs
C : cytosine
CACNA1A : calcium channel, voltage-dependent, P/Q type, alpha 1A subunit
cDNA : complementary DNA
CLC-1 : Chloride channel 1
CNBP : CCHC-type zinc finger, nucleic acid binding protein
CUG-BP : CUG binding protein
da : daughterless
DAPI : 4'-6-diamido-2-phenylindole
DIC : differential interference contrast
DM1 : myotonic dystrophy type 1
DM2 : myotonic dystrophy type 2
DMPK : dystrophia myotonica protein kinase
DNA : deoxyribonucleic acid
dNTP : deoxyribonucleoside triphosphate
DRPLA : dentatorubral-pallidoluysian atrophy
dsRNA : double-stranded RNA
DTT : dithiothreitol
EDTA : ethylene diamine tetra-acetic acid
elav : embryonic lethal abnormal vision
ERG : electroretinogram
FMR1 : fragile X mental retardation 1
FXTAS : fragile X tremor-ataxia syndrome
G : guanosine
GFP : green fluorescent protein
GMR : Glass multimer reporter
HD : Huntington’s disease
HDL-2 : Huntington’s disease like 2
hnRNP : heterogenous nuclear ribonucleoprotein
HTT : huntingtin
JPH3 : juntophilin 3
kb : kilobase
Abstract

The expansion of polymorphic repeat sequences within unrelated genes is responsible for pathology in a family of dominant human diseases. Based on clinical and genetic similarities, it is hypothesised that common pathways may contribute to all of these diseases, with evidence for a number of mechanisms mediated by the expanded repeat. Where the repeats are translated, a long polyglutamine protein has been shown to have pathogenic properties. However, the identification of diseases caused by untranslated repeats has led to the discovery of repeat RNA-mediated pathogenic pathways. As expanded repeat-containing transcripts are present in the case of both translated and untranslated repeats, repeat RNA is a candidate common pathogenic agent. Therefore, determining its contributions to pathology will be important in understanding these diseases.

Using the model organism Drosophila melanogaster, this study identifies common CUG and CAG repeat RNA-mediated phenotypes, enabling the investigation of common pathways of cellular perturbation. Ubiquitous expression of either repeat sequence led to reduced viability and disruption to the development of the adult dorsal abdominal tergites through a specific effect on histoblast cells. This phenotype provides a biological read-out of common RNA-mediated effects, enabling examination of the pathways involved by quantifying the changes in the phenotype when specific candidate genes are genetically altered. Tergite disruption was not strongly modified by reducing activity of the well-characterised muscleblind mediated pathway. Furthermore, the presence of specific nuclear RNA foci, an indicator of repeat RNA-mediated protein sequestration, was not correlated with the phenotype. Results indicate that tergite disruption is not strongly dependent on muscleblind sequestration and may involve an alternative pathway. Ectopic expression of either repeat did not cause significant phenotypes in the eye, or neurons, except in the case of one fortuitous transgene insertion. In this case, bi-directional transcription of the repeat tract facilitated by an endogenous promoter was necessary for pathology, providing support for a novel pathway of pathology involving the formation of double-stranded RNA. Subsequent comparison of the pathways involved in hairpin-forming single stranded RNA, and bi-directional double-stranded RNA mediated phenotypes in Drosophila supports the existence of multiple distinct pathways that contribute to cellular perturbation.