The role of the exoribonuclease Pacman/Xrn1 in wing development in *Drosophila*

Joseph Alexander Waldron

A thesis submitted in partial fulfilment of the requirements of the University of Brighton and the University of Sussex for the degree of Doctor of Philosophy at the Brighton and Sussex Medical School

December 2014
Abstract

RNA stability plays a critical role in the control of gene regulation by determining the levels of mRNA that can be translated into protein. The aim of this thesis is to investigate the role of the exoribonuclease Pacman in regulating gene expression during wing development in Drosophila melanogaster. Pacman is the only known cytoplasmic exoribonuclease which degrades RNA in a 5'-3' direction and is highly conserved across all eukaryotes.

The main phenotype of null pacman mutants is that the wing imaginal discs are significantly smaller than wild-type. The wing imaginal discs are the parts of tissue in the larvae which go on to form the adult wings and are first specified in the embryo. They are an excellent model system to study the growth and development of a multi-cellular organism as the pathways involved are highly conserved across eukaryotes, including humans.

Results from this thesis show that Pacman is required for the correct growth and development of the wing imaginal discs. The reduced size of the wing imaginal discs in pacman mutants was shown to be a direct consequence of the lack of cytoplasmic 5'-3' exoribonuclease activity within these cells. In addition to the mutant discs being smaller in size they were also delayed in development. Knocking down pacman using RNAi, within specific regions of the disc, also leads to smaller wings and wing vein defects.

Using mosaic analysis, it was shown that wing imaginal disc cells have a cell autonomous requirement for Pacman. The cause of the reduced growth and delayed development of pcm14 wing imaginal discs was shown to be an increase in apoptosis. Interestingly, compensatory proliferation was also shown to be occurring in these mutant discs, but clearly this was unable to compensate for the amount of apoptosis occurring.

A genetic interaction was observed between pacman and the intrinsic apoptosis pathway in Drosophila. In order to determine whether Pacman was regulating apoptosis directly through this pathway, q-RT-PCR was performed, to determine whether any of these pro-apoptotic genes were post-transcriptionally up-regulated. Results showed that both reaper and hid were significantly up-regulated, by 8 and 2 fold respectively, in pcm14 wing imaginal disc cells, yet levels of pre-reaper and pre-hid were not significantly different to wild-type. This therefore strongly supports the current hypothesis that Pacman is regulating apoptosis in Drosophila wing imaginal discs by regulating the expression of reaper and hid.
Candidates declaration

I declare that the research in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for degree.

Signed: [Signature]

Dated: 01/12/2014
Acknowledgements

I would first of all like to thank my supervisor, Sarah Newbury, both for the opportunity, but most importantly for the unequivocal support throughout my PhD. I am very grateful for her genuine interest and guidance, both personally and professionally.

I would also like to thank all members of the lab, both past and present, for all the help and support during my PhD and also for being great friends and colleagues.

I would particularly like to thank Dom Grima for the inspiration and supervision during the start of my PhD.

Chris Jones deserves a very large thank you for taking over the role of Dom in being my first point of call for any questions/disasters encountered and for the insightful discussions about my work.

Maria Zabolotskaya also deserves a big thank you for all the technical skills I have learned and for teaching me the importance of accuracy and tidiness.

I would also like to thank Ben Towler for his keen mutual interest both in my work and in football related matters and also to Clare Rizzo-Singh and Karen Scruby for their often overlooked but never unappreciated support.

I would also like to thank Juan Pablo Couso, Claudio Alonso and Robert Ray, in addition to all those who attended the Drosophila retreats over the years, for their critical feedback and enthusiasm.

Finally and most importantly, I would like to thank all my family and friends for always being there for me and especially to my partner Lauren. Without her constant love and support this journey would not have been possible. Together we made it!
Contents

1 Introduction ............................................................................................................. 1

1.1 Regulation of gene expression controls the balance of proteins within the cell .... 1

1.1.1 The importance of gene expression ................................................................. 1

1.1.2 The complexities of gene expression ............................................................... 2

1.1.2.1 Transcriptional regulation ......................................................................... 2

1.1.2.2 Post-transcriptional regulation .................................................................. 4

1.2 The role of mRNA stability in regulating gene expression .............................. 7

1.2.1 mRNA degradation pathways in eukaryotes ................................................ 7

1.2.1.1 Deadenylation dependent decay ............................................................... 7

1.2.1.2 Deadenylation independent decay ............................................................ 14

1.2.2 P bodies and Stress granules ......................................................................... 16

1.2.3 Quality control mechanisms ......................................................................... 18

1.2.3.1 Nonsense Mediated Decay (NMD) .......................................................... 18

1.2.3.2 Non Stop Decay (NSD) ............................................................................ 19

1.2.3.3 No Go Decay (NGD) ................................................................................ 19

1.2.4 Regulating mRNA half life ............................................................................ 20

1.2.4.1 ARE (AU-Rich Element) mediated decay ................................................. 20

1.2.4.2 microRNAs ............................................................................................... 22

1.2.5 The importance of RNA stability in development and disease .................. 26

1.2.5.1 Role of mRNA stability in development .................................................. 26

1.2.5.2 Diseases associated with RNA stability defects ...................................... 27

1.3 Advantages of studying RNA stability in Drosophila melanogaster ............... 29

1.3.1 Using Drosophila melanogaster as a model organism ................................... 29

1.3.2 Growth and development of Drosophila melanogaster ............................... 30

1.3.2.1 Life cycle of Drosophila melanogaster ..................................................... 30

1.3.2.2 Hormonal control of development ......................................................... 30

1.3.2.3 Patterning of the embryo ........................................................................ 31

1.3.3 RNA degradation pathways in Drosophila melanogaster ............................ 32
1.4 Using wing imaginal discs to study the regulation of growth and development ..36

1.4.1 Cell fate determination ................................................................. 36
1.4.2 Hormonal control of developmental timing ........................................ 37
1.4.3 Regulation of growth by the Hippo pathway ..................................... 39
1.4.4 Regulation of growth through mechanical force .................................. 40
1.4.5 Cell competition ........................................................................... 40
1.4.6 Termination of growth ..................................................................... 41

1.5 Background information on the role of Xrn1/Pacman in Drosophila melanogaster development ...................................................................................................................... 42

1.5.1 Structure and conservation of Xrn1 ................................................... 42
1.5.2 Phenotypes of xrn1 mutants and redundancy between the 5'-3' and 3'-5' pathways 45
1.5.3 Expression of Xrn1 during Drosophila development .......................... 46
1.5.4 Work leading up to this project ....................................................... 47

1.6 Aims of this project ............................................................................. 47

2 Materials and Methods ......................................................................... 49

2.1 Drosophila stocks ............................................................................. 49
2.2 Drosophila husbandry ......................................................................... 52

2.2.1 Drosophila food recipe ................................................................. 52
2.2.2 Stock maintenance ......................................................................... 52
2.2.3 Cross timings .................................................................................. 52
2.2.4 Virgin collection ............................................................................. 52
2.2.5 Staging/selecting larvae .................................................................. 53
2.2.6 Balancer chromosomes and genetic markers ..................................... 53
2.2.7 L3 imaginal disc dissection for size measurements ............................ 55
2.2.8 L3 wing imaginal disc dissection for RNA extraction and Western Blot ...... 55
2.2.9 L3 survival ....................................................................................... 55
2.2.10 Wing measurements ................................................................. 55
2.2.11 Larval weight and size measurements .................................. 56
2.2.12 Larval time measurements ..................................................... 56
2.3 Genetic techniques ................................................................. 57
  2.3.1 GAL4/UAS system ................................................................. 57
  2.3.2 GAL80ts system ................................................................. 57
  2.3.3 Mosaic analysis ................................................................. 59
    2.3.3.1 G418 screen ................................................................. 59
    2.3.3.2 Inducing mitotic recombination ........................................ 59
2.4 PCR/sequencing .................................................................. 60
  2.4.1 Quick DNA extraction ......................................................... 60
  2.4.2 PCR primer design ............................................................. 60
  2.4.3 PCR on genomic DNA ......................................................... 60
  2.4.4 Agarose gel electrophoresis ................................................ 60
  2.4.5 Gel extraction of PCR products/DNA sequencing .................. 61
  2.4.6 Mapping/aligning sequence ................................................. 61
  2.4.7 Primers used ..................................................................... 61
2.5 Quantitative RT-PCR ............................................................. 63
  2.5.1 RNA extraction ................................................................. 63
  2.5.2 cDNA production .............................................................. 63
  2.5.3 q-PCR ............................................................................ 63
2.6 Western blotting ................................................................. 65
  2.6.1 Sample preparation ............................................................. 65
  2.6.2 Gel separation/blotting ......................................................... 65
  2.6.3 Developing and quantification ............................................. 66
  2.6.4 Antibodies ..................................................................... 66
2.7 Immunocytochemistry .......................................................... 67
2.7.1 Slide preparation/fixing the cells ................................................................. 67
2.7.2 Staining/washes .......................................................................................... 67
2.7.3 Antibodies .................................................................................................... 67
2.7.4 Confocal microscopy/calculating the mitotic index ..................................... 68
2.8 cRACE .............................................................................................................. 69
  2.8.1 Shrimp alkaline phosphatase (SAP) ............................................................ 69
  2.8.2 Tobacco acid pyrophosphatase (TAP) ....................................................... 69
  2.8.3 RNA ligation ............................................................................................... 70
  2.8.4 Reverse Transcription ................................................................................ 70
  2.8.5 PCR ............................................................................................................... 71
  2.8.6 Cloning ......................................................................................................... 72
    2.8.6.1 Ligation .................................................................................................. 72
    2.8.6.2 Transformation ...................................................................................... 72
    2.8.6.3 Inoculation ............................................................................................. 72
    2.8.6.4 Mini preps/sequencing .......................................................................... 72
2.9 Statistical analyses .......................................................................................... 73

3 Characterising the $pcm^{14}$ mutant phenotypes ........ 74
  3.1 Introduction ....................................................................................................... 74
  3.2 Previous Work .................................................................................................. 74
    3.2.1 Characterisation of the pacman alleles ..................................................... 74
    3.2.2 Genetic evidence that $pcm^{14}$ is a null mutant ...................................... 77
    3.2.3 Molecular evidence that $pcm^{14}$ is a null mutation ................................. 79
    3.2.4 Phenotypes of the pacman mutants ....................................................... 83
  3.3 Aims ............................................................................................................... 84
  3.4 Pacman is expressed ubiquitously throughout the wing imaginal disc .......... 85
  3.5 Pacman expression is essential for Drosophila development ....................... 85
The exoribonuclease activity of Pacman is essential for *Drosophila* development

*pcm*\(^{14}\) wing imaginal discs are significantly smaller than wild-type due to a lack of Pacman expression in the wing imaginal disc cells.

The exoribonuclease activity of Pacman is essential for correct wing imaginal disc development.

Knockdown of *pacman* expression in wing imaginal disc cells using RNAi does not reduce the size of the wing imaginal discs.

Knockdown of *pacman* expression in specific domains of the wing disc results in loss of tissue in the corresponding domains of the adult wing.

Leg, eye and haltere imaginal discs are significantly smaller in *pcm*\(^{14}\) L3 larvae compared to wild-type.

*pcm*\(^{14}\) larvae are not significantly smaller than wild-type.

*pcm*\(^{14}\) larvae are delayed in development.

*pcm*\(^{14}\) wing imaginal disc development is delayed.

*pcm*\(^{14}\) larvae are delayed in development.

*pcm*\(^{14}\) wing imaginal disc development is delayed.

*pacman* expression is critical for wing imaginal disc development during early L3.

Pacman expression is not required during embryogenesis or L1 for the viability of the organism.

Chapter summary.

Acknowledgements.

Chapter discussion.

Why is *pcm*\(^{14}\) a null allele?

Are the neighbouring genes *CR43260* and *Nat1* affected by the *pcm*\(^{14}\) mutation?

Why are wing imaginal discs not smaller when *pacman* is knocked down using RNAi?

Expressing a "nuclease dead" Pacman throughout the wing imaginal discs in *pcm*\(^{14}\) larvae has a dominant negative effect on the wing imaginal disc phenotype.
4 Investigating the reduced growth phenotype of \textit{pcm}^{14} \textit{wing} imaginal discs ................................................................. 125

4.1 Introduction .................................................................................................................. 125

4.2 Aims............................................................................................................................... 128

4.3 Creating the \textit{w}^{1118} \textit{pcm}^{14} \textit{P[neoFRT]19A/FM7i} stock ........................................... 128

4.4 Populations of \textit{pcm}^{14} \textit{wing} imaginal disc cells have reduced growth when compared to populations of wild-type \textit{wing} imaginal disc cells ........................................................................ 133

4.5 Loss of Pacman induces ectopic apoptosis in the \textit{wing} pouch region of the \textit{wing} imaginal discs .................................................................................................................. 135

4.6 The \textit{pcm}^{14} mutation results in compensatory proliferation of \textit{wing} imaginal disc cells 138

4.7 Chapter summary........................................................................................................... 141

4.8 Chapter discussion ......................................................................................................... 141

4.8.1 Why is apoptosis restricted to the \textit{wing} pouch of the discs? ............................. 141

4.8.2 Why is the compensatory proliferation not sufficient to counteract the increased apoptosis? .................................................................................................................. 142

4.8.3 Why is the compensatory proliferation not concentrated to \textit{cells} neighbouring the apoptotic cells? ............................................................................. 143

4.8.4 Is Caspase 3 staining a reliable indication of apoptotic cells? ............................ 144

5 How is Pacman regulating apoptosis in the \textit{wing} imaginal discs? ................................................................. 146

5.1 Introduction .................................................................................................................. 146

5.1.1 Programmed cell death (Apoptosis) .................................................................... 146

5.1.2 Intrinsic apoptosis pathway in \textit{Drosophila} ......................................................... 150

5.1.3 Conservation of the apoptosis pathways in \textit{Drosophila} and mammals .............. 152

5.1.4 Releasing the brakes or stepping on the gas? ..................................................... 153

5.1.5 Regulation of apoptosis in \textit{Drosophila} ................................................................ 153
5.2 Aims.......................................................................................................................... 155

5.3 Inhibition of apoptosis partially rescues the reduced growth of pcm\textsuperscript{14} wing imaginal disc cells.......................................................................................................................... 156

5.4 The delayed development of pcm\textsuperscript{14} wing imaginal discs is rescued by inhibiting apoptosis.......................................................................................................................... 159

5.5 Inhibiting apoptosis fails to rescue pcm\textsuperscript{14} pupal lethality.......................... 161

5.6 Is Pacman specifically degrading reaper, hid or grim mRNA? ....................... 163
  5.6.1 reaper is up-regulated post-transcriptionally in pcm\textsuperscript{14} wing imaginal disc cells 165
  5.6.2 hid is post-transcriptionally up-regulated in pcm\textsuperscript{14} wing imaginal disc cells 168
  5.6.3 grim was not significantly up-regulated in pcm\textsuperscript{14} wing imaginal disc cells.. 168

5.7 Chapter summary.................................................................................................... 170

5.8 Chapter discussion............................................................................................... 170
  5.8.1 Is Reaper and Hid protein increased in pcm\textsuperscript{14} wing imaginal discs?....... 170
  5.8.2 Are any additional apoptotic genes mis-expressed in pcm\textsuperscript{14} mutant wing imaginal disc cells?.................................................................................................................. 171

6 Is reaper a direct target of Pacman? .............................................. 173

6.1 Using circularised rapid amplification of cDNA ends (cRACE) to identify the 5' and 3' ends of reaper transcripts.................................................................................................................. 175

6.2 reaper mRNA is transcribed from position -16......................................................... 180

6.3 Identifying capped and decapped transcripts ...................................................... 182

6.4 Wild-type and pcm\textsuperscript{14} capped reaper transcripts lack the 3' end of the mRNA and do not have poly(A) tails ............................................................................................................. 184

6.5 Wild-type and pcm\textsuperscript{14} decapped reaper transcripts have incomplete 5' and 3' ends 187

6.6 Chapter summary.................................................................................................... 191

6.7 Chapter discussion............................................................................................... 191
  6.7.1 Are reaper transcripts undergoing 3'-5' decay ............................................. 191
7 Discussion........................................................................................................ 192

7.1 Summary of main findings ............................................................................. 192

7.1.1 Characterisation of pcm<sup>14</sup> phenotypes ............................................. 192

7.1.2 Reduced growth of the wing imaginal discs is caused by an increase in apoptosis 192

7.1.3 Loss of Pacman results in a post-transcriptional up-regulation in reaper and hid mRNA ........................................................................................................ 193

7.2 Is Pacman directly regulating apoptosis? ...................................................... 194

7.3 Are reaper and hid direct targets of Pacman? ................................................ 198

7.3.1 Are reaper and hid directly targeted to Pacman through cis elements within their 3'UTRs ........................................................................................................ 198

7.3.2 Do reaper and hid require degradation by the 5'-3' pathway or can the 3'-5' pathway compensate .......................................................................................... 201

7.4 Are the increased levels of reaper and hid translated? ................................. 201

7.4.1 Are increased levels of reaper and hid polyadenylated? ............................. 201

7.4.2 Pacman is able to act as a decapping factor ............................................ 202

7.4.3 reaper and hid are able to undergo cap independent translation .......... 205

7.5 Reaper is involved in global shutdown of translation...................................... 206

7.7 Developmental parameters of apoptosis ........................................................ 207

7.9 Implications for human disease ....................................................................... 208

7.9.1 Is the regulation of RNA stability critical in preventing cancer? .............. 208

7.10 Future work ................................................................................................... 211

7.10.1 Measuring the half life of reaper .............................................................. 211

7.10.2 Using tissue culture to further investigate the regulation of reaper and hid by Pacman 211

7.10.2.1 cRACE ................................................................................................. 212

7.10.2.2 Polysome profiling ............................................................................. 212
7.10.2.3 Using 3'UTR reporters to determine whether Pacman regulates reaper and hid through their 3'UTRs ................................................................. 213

7.10.3 Investigating the role of Pacman in additional Drosophila systems .......... 213

7.11 Concluding remarks .................................................................................. 214

8 References ........................................................................................................ 215
Table of figures

Figure 1.1. An overview of gene expression: from transcription through to protein degradation................................................................. 3

Figure 1.2. Deadenylation dependent decay......................................................... 11

Figure 1.3. ARE mediated decay. ............................................................... 21

Figure 1.4. miRNA mediated repression occurs in a stepwise manner, whereby translational repression is followed by deadenylation and degradation by the 5′-3′ machinery. .......... 25

Figure 1.5. Wing imaginal disc fate map............................................................. 38

Figure 1.6. Structure and conservation of Xrn1................................................. 43

Figure 2.1. Area of the wing measured for size.................................................. 58

Figure 2.2. Diagrammatic representation of the GAL4/UAS and the GAL80ts system....... 58

Figure 2.3. Primers used to detect different pacman alleles/constructs. ..................... 62

Figure 3.1. Diagrammatic representation of the pacman alleles............................. 75

Figure 3.2. Genetic evidence that pcm5 is a hypomorphic mutation and pcm14 is a null mutation. ................................................................................. 78

Figure 3.3. Estimation of the relative function of Pacman protein produced from the pacman alleles in vivo ............................................................ 82

Figure 3.4. Pacman is expressed throughout the wing imaginal disc......................... 86

Figure 3.5. Generation of the y′ pcm14/ FM7i-GFP; UAS-pcmWT/Cyo-GFP and the pcm14/ FM7i-GFP; UAS-pcmKO/Cyo-GFP stocks................................................................. 87

Figure 3.6. pcm14 pupal lethality can be rescued by expressing UAS-pcmWT in pcm14 larvae with different GAL4 drivers................................................................. 87

Figure 3.7. 69B-GAL4 driven expression of UAS-pcmWT rescues pcm14 lethality 100% at 25°C. ............................................................................................................. 91

Figure 3.8. Diagrammatic representation of the expression patterns for the GAL4 drivers used.............................................................................................................. 95

Figure 3.9. Expression of pacman throughout the wing imaginal discs of pcm14 larvae rescues the reduced size of wing imaginal discs................................................. 97
Figure 3.10. Wing imaginal disc size is not reduced when pacman is knocked down using RNAi. ................................................................. 100

Figure 3.11. Pacman is effectively knocked down in 69B-GAL4/UAS-pcmRNAi wing imaginal discs........................................................................................................ 100

Figure 3.13. Knockdown of Pacman in specific compartments of the wing imaginal disc causes reduced growth and wing venation phenotypes in the corresponding compartment of the wing. ........................................................................................................ 103

Figure 3.14. pcm14 L3 larvae have significantly smaller metathoracic leg, haltere and eye imaginal discs................................................................. 104

Figure 3.15. pcm14 larvae are not significantly smaller than pcmWT larvae, but are delayed in development................................................................. 106

Figure 3.16. Wing imaginal disc development in pcm14 larvae is morphologically delayed by 32 hours as determined by Wingless staining. ......................................... 108

Figure 3.17. Pacman is effectively knocked down in en-GAL4/+; UAS-pcmRNAi/GAL80ts larvae 24-32h after temperature increase from 19°C to 29°C........................................ 110

Figure 3.18. Diagrammatic representation of the temperature shifts carried out during the GAL80ts experiment. ................................................................. 112

Figure 3.19. Wing size is reduced and wing venation phenotypes are observed only in flies in which Pacman has been knocked down before late L3......................................... 114

Figure 3.20. Pacman expression is critical during early L3 for correct wing development to occur. ........................................................................................................ 115

Figure 3.21. Pacman expression is not required during embryogenesis or L1 for the viability of the organism. ........................................................................... 117

Figure 3.22. Annotated screen shot of RNA-Seq results showing mapped regions of transcripts at the pacman locus for pcmWT and pcm14 poly(A) selected wing imaginal disc RNA. ........................................................................... 120

Figure 4.1. Diagrammatic representation showing the principles of mosaic analysis. ....... 127

Figure 4.2. Creation of the w118 pcm14 P(neoFRT19A)/FM7i stock. ..................................... 129

Figure 4.3. Confirmation of the presence of the pcm14 mutation in the w118 pcm14 P(neoFRT)19A/FM7i stock. ................................................................. 132
Figure 4.4. Populations of wild-type cells have a significant growth advantage over *pcm*<sup>14</sup> cells. ............................................................................................................................................. 134

Figure 4.5. Ectopic apoptosis occurs throughout and specific to the wing pouch region of the wing imaginal discs in *pcm*<sup>14</sup> L3 larvae. ............................................................................................................................................. 136

Figure 4.6. Knockdown of *pacman* using RNAi causes apoptosis specifically in the wing pouch region of the wing imaginal discs in L3 larvae. ............................................................................................................................................. 137

Figure 4.7. The mitotic index is greater in *pcm*<sup>14</sup> wing imaginal discs compared to wild-type. ............................................................................................................................................. 140

Figure 5.1. Diagrammatic representation of the intrinsic cell death pathways in *Drosophila* and mammals............................................................................................................................................. 149

Figure 5.2. Diagrammatic representation of the crosses carried out in order to inhibit apoptosis in *pcm*<sup>14</sup> L3 larvae. ............................................................................................................................................. 157

Figure 5.3. Inhibiting apoptosis partially rescues the size of the *pcm*<sup>14</sup> wing imaginal discs. ............................................................................................................................................. 158

Figure 5.4. Inhibiting apoptosis rescues the delay in *pcm*<sup>14</sup> wing imaginal disc development. ............................................................................................................................................. 160

Figure 5.5. Inhibiting apoptosis in *pcm*<sup>14</sup> developing larvae does not rescue pupal lethality. ............................................................................................................................................. 162

Figure 5.6. Diagrammatic representation of the custom TaqMan primer/probe sets used to measure the pre-mRNAs of (A) *hid* and (B) *reaper* and (C) *grim*. ............................................................................................................................................. 164

Figure 5.7. *reaper* is up-regulated in *pcm*<sup>14</sup> wing imaginal disc cells mainly at the post-transcriptional level. ............................................................................................................................................. 166

Figure 5.8. *hid* is post-transcriptionally up-regulated in *pcm*<sup>14</sup> wing imaginal disc cells compared to wild-type............................................................................................................................................. 169

Figure 5.9. *grim* is lowly expressed and not significantly up-regulated in *pcm*<sup>14</sup> wing imaginal disc cells compared to wild-type. ............................................................................................................................................. 169

Figure 6.1. A diagrammatic overview of circularised rapid amplification of cDNA ends (cRACE). ............................................................................................................................................. 176

Figure 6.2. Location of *reaper* primers used for the reverse transcription and PCR. ............................................................................................................................................. 177

Figure 6.3. Amplified cRACE PCR products. ............................................................................................................................................. 179
Figure 6.4. Sequence alignment of the published reaper sequence taken from FlyBase and the actual sequence of reaper transcripts sequenced during the cRACE experiment. ......................................................... 181

Figure 6.5. pcm14 transcripts from the capped pool which lack a complete 5’ end are most likely decapped transcripts. .................................................................................................................. 183

Figure 6.6. Both wild-type and pcm14 capped transcripts lack complete 3’ ends. ....................... 186

Figure 6.7. Both wild-type and pcm14 decapped transcripts lack complete 5’ and 3’ ends. 188

Figure 6.8. Scatter plots showing transcript length, 5’ most end and 3’ most end of all the transcripts sequenced. Error bars represent standard deviation.......................................................... 190

Figure 7.1. Model of the hypothesis that Pacman is indirectly required in pcm14 wing imaginal disc cells to prevent apoptosis occurring. ................................................................. 196

Figure 7.2. Model of the hypothesis that Pacman is directly required to degraded endogenous levels of reaper and hid in order to prevent apoptosis. ............................................. 196

Figure 7.3. Diagrammatic representation of the 3’UTRs for (A) reaper and (B) hid, showing predicted and validated miRNA binding sites taken from TargetScanFly release 6.2. .......... 200

Figure 7.4. Hypothetical model explaining the dominant negative effect of nuclease dead Pacman overexpression by its role as a decapping factor................................................................. 204

7.5. Diagrammatic representation of the hypothesis being tested in the Newbury lab explaining why pacman is expressed at low levels in several osteosarcoma cell lines. ....... 210
## Commonly used abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>XRN1/Xrn-1/Xrn1p/Pacman</td>
<td>Exoribonuclease-1</td>
</tr>
<tr>
<td>Dcp1/2</td>
<td>Decapping protein 1/2</td>
</tr>
<tr>
<td>PABP</td>
<td>Poly(A) Binding Protein</td>
</tr>
<tr>
<td>P bodies</td>
<td>Processing bodies</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>pre-mRNA</td>
<td>preliminary messenger RNA</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic DNA</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>RBP</td>
<td>RNA Binding Protein</td>
</tr>
<tr>
<td>ARE</td>
<td>AU Rich Element</td>
</tr>
<tr>
<td>5'UTR</td>
<td>5' Untranslated Region</td>
</tr>
<tr>
<td>3'UTR</td>
<td>3' Untranslated Region</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal Ribosome Entry Site</td>
</tr>
<tr>
<td>NMD</td>
<td>Nonsense Mediated Decay</td>
</tr>
<tr>
<td>q-RTPCR</td>
<td>Quantitative Reverse Transcriptase PCR</td>
</tr>
<tr>
<td>cRACE</td>
<td>Circularised Rapid Amplification of cDNA Ends</td>
</tr>
<tr>
<td>AEL</td>
<td>After Egg Lay</td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream Activation Sequence</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Regulation of gene expression controls the balance of proteins within the cell

1.1.1 The importance of gene expression

Since the discovery of DNA and the birth of genetics, it is widely accepted that the code for life is encrypted within DNA. Within the DNA sequence of any organism are several thousand genes, each encoding a single protein or RNA structure. The entire sequence of nucleotides within the cell is termed the genome and this is arranged into chromosomes which are stored within the nucleus of almost every cell. These chromosomes can be thought of as the cells "master copy" of genetic material, as an exact replica of this sequence is passed on from cell to cell during replication.

This sequence of DNA is therefore of crucial importance for every cell and it is not surprising that there are many surveillance mechanisms that aim to prevent any DNA damage or genomic instability. Indeed, mutations to the DNA sequence are the primary cause of many diseases including cancer. However, despite the obvious paramount importance of genome integrity, the role of DNA to encode proteins and RNAs is actually limited to the first step of gene expression, known as transcription. The first step in which a gene encodes a protein involves the DNA sequence being transcribed into a complimentary sequence of RNA, termed messenger RNA (mRNA). This occurs in the nucleus, and is then exported into the cytoplasm once properly processed. Once inside the cytoplasm the mRNA recruits the translational machinery, including the ribosomes, so that the RNA sequence can then be converted into a sequence of amino acids. This sequence of amino acids then folds into a predetermined structure to form the protein. This is a very simplified overview of how a single gene encodes for a single protein and it is far more complicated than this in reality. However, this overview highlights the importance of RNA for the controlled regulation of gene expression. This flow of information from DNA to RNA to protein is crucial in preserving genomic integrity and was termed the central dogma of molecular biology by Francis Crick (Crick, 1970).

In order for a cell to function correctly, it requires the right proteins and RNAs to be present in the right amounts and in the correct location. In view of the fact that each somatic cell in a multicellular organism contains an identical DNA sequence, how is it that
each cell type has a distinct function and morphology? The mechanism by which cells achieve this is by regulating which genes are switched on (expressed), and by how much. It is now clear that the regulation of gene expression is essential for controlling the fate of cells, as every cell type has a unique signature of which genes it expresses and at what levels. Changes in gene expression are essential during the development of multicellular organisms and also for cells to adapt to stress and the environment. There are many steps involved in expressing genes as functional proteins and it used to be thought that the majority of regulation occurred at the level of transcription. However, this view is now outdated, as it is clear that each step of gene expression is under tight regulation so that each gene is expressed in the correct spatial and temporal pattern (Garneau et al, 2007). A simplistic overview of the steps involved in producing a functional protein from a single gene is shown in Figure 1.1. and the complexities involved will be discussed in more detail below.

### 1.1.2 The complexities of gene expression

#### 1.1.2.1 Transcriptional regulation

As mentioned above, the first step of gene expression requires the DNA sequence to be transcribed into a mRNA transcript. Regulation of transcription can occur at the level of initiation (Kadonaga, 2004; Lelli et al, 2012), elongation (Gaertner & Zeitlinger, 2014; Nechaev & Adelman, 2008; Selth et al, 2010) and termination (Mischo & Proudfoot, 2013; Proudfoot, 2011; Richard & Manley, 2009; West & Proudfoot, 2009). The mechanisms which regulate transcription initiation rely on cis elements within the DNA sequence, such as promoter and enhancer sequences and trans elements present within the cell, such as transcription factors (Kadonaga, 2004). A further complexity of transcriptional initiation involves access of RNA Polymerase to the DNA which is tightly compacted and interwoven with chromatin. Protein modifications are able to further promote of repress transcription initiation by regulating the affinity between the DNA and the histones (Kouzarides, 2007; Li et al, 2007). This alteration of the transcriptional potential of a gene, without altering the sequence of the DNA, can be inherited through cell divisions and is termed epigenetics.
Figure 1.1. An overview of gene expression: from transcription through to protein degradation.

In order for a functional protein to be produced from a gene, the gene must first be transcribed to produce the pre-mRNA. This is then processed by removing the introns and adding a m\(^7\)G cap to the 5’ end of the transcript and a poly(A) tail to the 3’ end. Once inside the cytoplasm the mRNA circularises through a direct interaction with eIF4G which binds both the cap binding protein eIF4E and poly(A) binding protein (PABP). Circularisation of the mRNA allows for efficient translation. mRNAs not undergoing active translation can either be stored in a translationally repressed state, or degraded. Once the protein is translated its function is still under regulation from post-translational modifications, such as ubiquitination, which targets the modified protein to the proteosome.
Processing of the pre-mRNA occurs co-transcriptionally (Moore & Proudfoot, 2009), with many protein complexes binding to and dissociating with the mRNA, forming a mRNA Protein (mRNP) complex which is essential for nuclear export (Iglesias & Stutz, 2008; Kelly & Corbett, 2009). The three main processing events required for the maturation and export of almost all mRNAs are; 1) the addition of a methylated guanine (m7G) cap to the 5' end of the mRNA via a unique 5' to 5' triphosphate linkage, 2) splicing out of the introns and 3) the addition of a poly(A) tail to the 3' end of the mRNA. These processing steps are interwoven, both with each other and also to upstream events in transcription and downstream events in translation and mRNA decay (Moore & Proudfoot, 2009). In addition to these events regulating the final output of protein levels they can also be regulated to produce alternative transcripts from the same gene. This is achieved either through alternative splicing (Chen & Manley, 2009) or alternative cleavage and polyadenylation (Di Giammartino et al, 2011; Tian & Manley, 2013). Alternative splicing causes alternative transcripts to be produced, which can have different functions due to the difference in amino acid sequence. Alternative polyadenylation is more often associated with differential regulation of the mature mRNA through the regulatory regions within the 3'UTR, which can be either included or excluded. This allows an mRNA to either gain or loss the regulation imposed on them by trans factors such as microRNAs and RBPs. This has been shown to have the potential to have dramatic consequences on protein levels, both in the normal development of stem cells (Mueller et al, 2013) and also during the development of diseases such as cancer (Mayr & Bartel, 2009).

### 1.1.2.2 Post-transcriptional regulation

Once the mRNP is exported into the cytoplasm the mRNA forms a circular conformation through the interactions between the 5’ and 3’ ends. This is caused by a direct interaction with eIF4G which binds both the cap binding protein eIF4E and poly(A) binding protein (PABP) which are bound to the 5’ and 3’ ends of the mRNA respectively. Circularisation of the mRNA allows for efficient translation and mRNAs undergoing active translation are found associated with multiple ribosome complexes. Similar to transcription, translation is also regulated at the stages of initiation, elongation and termination, although it is thought that the majority of regulation occurs at the level of initiation (Jackson et al, 2010). In order for translation initiation to occur, several additional eukaryotic Initiation Factors (eIFs) are recruited to the mRNP to form the 48S initiation complex. eIF4G acts as a scaffold, binding to the cap binding protein eIF4E and also eIF4A to form the eIF4F complex. In addition to its
role in circularisation, the elf4F complex is involved in unwinding the 5’UTR secondary structure and recruiting the 43S pre-initiation complex through binding to elf3. The 43S pre-initiation complex is composed of the 40S ribosomal subunit, the elf2-GTP-Met-tRNA\textsuperscript{Met} ternary complex, elf3, elf1, elf1A and elf5. This 48S initiation complex then scans the 5’UTR until it finds a suitable start codon (most likely AUG). Subsequent recruitment of the 60S ribosomal subunit allows formation of the elongation competent 80S ribosome and the dissociation of the elfs (Jackson et al, 2010; Sonenberg & Hinnebusch, 2009; Van Der Kelen et al, 2009).

Regulation of translation initiation can occur at multiple levels. The two main categories in which translation can be regulated are; those that affect the activity of elfs and/or ribosomes, which would regulate all cap dependent translation and those that regulate the translation of specific mRNAs. The main mechanism to inhibit global cap-dependent translation is through regulating the activity of elf2 and elf4F through phosphorylation. Phosphorylated elf2 is unable to be recycled, which leads to reduced levels of the elf2-GTP-Met-tRNA\textsuperscript{Met} ternary complex. This leads to a down-regulation in global translation levels, but actually allows for the selective translation of mRNAs with at least 2 upstream Open Reading Frames (uORFs) (Jackson et al, 2010).

Levels of functional elf4F complex can be regulated by phosphorylation of elf4E binding proteins (4E-BPs) by the mTOR pathway. When hypophosphorylated, these 4E-BPs are capable of competing with elf4G for the binding of elf4E, and so prevent formation of the elf4F complex and its recruitment to the 5’ m\textsuperscript{7}G cap of the mRNA (Jackson et al, 2010; Topisirovic et al, 2011). This therefore inhibits global levels of cap dependent translation. However, some mRNAs have evolved to initiate cap-independent translation through the presence of an Internal Ribosome Entry Site (IRES). These IRES sequences were first discovered in viruses, where it is thought the virus would shut down global cap dependent translation in order to hijack the cellular translational machinery to allow the translation of its IRES containing mRNAs. (Jackson et al, 2010; Le Quesne et al, 2010; Sonenberg & Hinnebusch, 2009; Van Der Kelen et al, 2009).

In order for the translation of specific mRNAs to be regulated, specific binding sites for RNA Binding Proteins (RBPs) and/or miRNAs are utilised. These cis elements are usually located within the 3’UTR of the mRNA and ultimately recruit protein complexes that regulate the fate of that specific mRNA. miRNA mediated repression will ultimately lead to translational repression, deadenylation and mRNA decay, although the contribution of these three mechanisms of repression is still under debate and will be discussed further later in this
The RNA binding proteins, cytoplasmic polyadenylation element (CPE) in *C. elegans* and Bruno in *Drosophila*, have been shown to repress translation of specific mRNAs through a similar mechanism to the 4E-BPs, in that they recruit the 4E-BP like proteins Maskin and Cup respectively to the mRNA. The specificity of this mechanism therefore arises from mRNAs which contain CPE and Bruno binding sites within their 3'UTRs. (Topisirovic et al, 2011). These mRNAs can be kept in a repressed state until they are later required.

The balance between whether an mRNA is translated or degraded is specific to each individual mRNA and is entirely dependent on the protein complexes that it is bound (Moore, 2005). The fate of an mRNA is therefore predetermined by the regulatory sequences within the mRNA and the cellular environment in which it is localised. In addition to translational regulation, mRNA stability also plays a vital role in determining how much mRNA can be translated into protein (Garneau et al, 2007; Newbury, 2006; Schoenberg & Maquat, 2012). Competition between translation and degradation occurs, which is not surprising when you consider that translation requires mRNA circularisation and mRNA degradation requires disruption of the circular conformation to gain access to either the 5' or 3' ends of the mRNA. Indeed, it is widely accepted that the first step in bulk eukaryotic mRNA degradation is deadenylation (Chen & Shyu, 2011; Wahle & Winkler, 2013), which removes the poly(A) tail and hence prevents PABP from being able to bind to the mRNP. Furthermore decapping is required for 5'-3' mRNA decay, demonstrating how the degradation and translational machinery communicate in order to determine the outcome of an mRNA.
1.2 The role of mRNA stability in regulating gene expression

The importance of mRNA stability is highlighted by the fact that the availability of a translationally competent mRNA is directly dependent on the balance between its rate of transcription and its rate of degradation. Despite this, it was first believed that mRNA degradation was not regulated but that it performed more of a "house keeping" role and that the level of regulation occurred at the rate of transcription. However, over the past 20 years, the importance of mRNA stability has been clearly demonstrated, and it is now generally accepted that mRNA stability plays a major role in the regulation of gene expression (Alonso, 2012; Arraiano et al, 2010; Garneau et al, 2007; Newbury, 2006; Newbury et al, 2006; Perez-Ortin et al, 2013; Reznik & Andersen, 2010; Schoenberg & Maquat, 2012). Furthermore, there are increasing examples of diseases that have developed as a result of impairment to the RNA degradation pathways (Reis et al, 2013). The focus of this thesis is to investigate the importance of RNA stability for the development of multicellular organisms.

The regulation of mRNA stability can be broadly categorised into two classes. The first of these classes is quality control. Surveillance mechanisms are in place to recognise and destroy mRNAs that if translated could cause toxicity to the cells. The second of these classes involves the regulation of mRNA half-life in order to alter the amount of mRNA which can be translated into protein. There is substantial overlap in the mechanisms of decay in which these two classes utilise, with specificity arising from the protein or RNA complexes which are involved in targeting the mRNAs to the degradation machinery. This chapter will begin with an overview of the RNA degradation pathways, the protein complexes involved and the mechanisms in which they are regulated. This will be followed by discussing the evidence which demonstrates the importance of RNA stability including the diseases which are associated with faulty RNA degradation machinery.

1.2.1 mRNA degradation pathways in eukaryotes

1.2.1.1 Deadenylation dependent decay

The RNA degradation pathways were first characterised in *S. cerevisiae*, with work from Roy Parker’s group providing a substantial insight into the mechanisms of RNA turnover (Parker, 2012). These initial studies indicated that bulk cytoplasmic mRNA decay begins with the shortening of the poly(A) tail (Decker & Parker, 1993; Muhlrad & Parker, 1992), followed by decapping and subsequent 5′-3′ degradation (Hsu & Stevens, 1993; Muhlrad et
al, 1994; Muhlrad et al, 1995), although deadenylated mRNAs can also be degraded 3'-5'
(Anderson & Parker, 1998). Although these studies indicated a preference for 5'-3' decay,
there is clear redundancy in that when the 5'-3' pathway is inhibited, mRNAs can also be
degraded by the 3'-5' pathway, although at significantly slower rates (Muhlrad et al, 1994).
The redundancy between the two pathways was highlighted by a synthetic lethality screen
which identified mutations in the exosome components Ski2 and Ski3 to be synthetic lethal
with mutations in Xrn1 (Anderson & Parker, 1998; Johnson & Kolodner, 1995). However,
the reduced growth phenotypes of Xrn1 mutants suggest that some mRNAs require the 5'-3'
pathway, perhaps due to the faster rates of turnover (Larimer & Stevens, 1990). Furthermore,
changes in levels of both steady state and decay rates of mRNAs were more apparent in mutant strains for components of the 5'-3' pathway (Beelman et al, 1996;
Dunckley & Parker, 1999).

Since these initial studies, it has been shown that mammalian cells also initiate mRNA
degradation with the shortening of the poly(A) tail (Couttet et al, 1997; Zheng et al, 2008)
and it is now widely accepted that bulk eukaryotic mRNA degradation is deadenylation
dependent (Chen & Shyu, 2011; Decker & Parker, 1994; Wahle & Winkler, 2013).

**Deadenylation**
The mechanism by which deadenylation occurs in eukaryotic cells is highly conserved.
Transcriptional pulse studies have indicated it to be a two-step mechanism, by which the
poly(A) tail is initially trimmed by the Pan2-Pan3 complex, followed by the action of the
Ccr4-Not complex, which is responsible for the majority of deadenylation in eukaryotic cells

**Ccr4-Not complex**
The Ccr4-Not complex is composed of five conserved canonical subunits, named Ccr4,
Caf1/Pop2, Not1, Not2 and Not3/5. The catalytic activity stems from the Mg$^{2+}$ dependent
poly(A) specific 3’ exonucleases Ccr4 and Caf1. The Not proteins are also crucial to the
complex, with Not1 providing a central scaffold. The Ccr4-Not complex is the predominant
deadenylase in eukaryotes and removes the poly(A) tail in a processive manner and its
activity is inhibited by PABP (Chen & Shyu, 2011; Goldstrohm & Wickens, 2008; Parker,
2012; Wahle & Winkler, 2013).

**Pan2/Pan3 complex**
The Pan2/Pan3 complex is a distributive deadenylase which is thought to be involved in the
initial trimming of the poly(A) tail. The enzymatic activity stems from the Pan2 protein and
in contrast to the Ccr4-Not complex, is stimulated by PABP through a direct interaction mediated by Pan3. Although the Pan2-Pan3 complex is not involved in the bulk deadenylation, it has been shown to be able to compensate for the loss of Ccr4-Not activity (Chen & Shyu, 2011; Parker, 2012; Wahle & Wickens, 2013).

PARN

In addition to the Ccr4-Not and Pan2-Pan3 complexes, higher eukaryotes have an additional deadenylase enzyme named poly(A) specific ribonuclease (PARN). PARN is stimulated by binding to the 5' m^7G cap and like the Ccr4-Not complex, is also inhibited by PABP (Goldstrohm & Wickens, 2008).

Diversity and redundancy of deadenylase enzymes

The diversity of deadenylase enzymes varies between species. Whereas humans have two paralogues of Ccr4, Caf1 and PARN, Drosophila has no PARN and only one Ccr4 and Caf1, yet Arabidopsis thaliana is thought to have as many as 26 deadenylases (Goldstrohm & Wickens, 2008). The redundancy between these enzymes is not yet fully understood. In S. cerevisiae it appears that Caf1 has lost its deadenylase activity (Parker, 2012) yet in Drosophila Caf1 is thought to be the predominant deadenylase enzyme in this complex (Temme et al, 2010). The paralogues of Ccr4 and Caf1 in higher eukaryotes are included into the complex in a mutually exclusive manner (Wahle & Wickens, 2013) and it is proposed that the increased complexity in higher eukaryotes allows certain deadenylases to target specific mRNAs (Aslam et al, 2009; Lee et al, 2012; Mittal et al, 2011) although the mechanism in which these mRNAs are targeted to specific deadenylases remains unclear.

In addition, there are more distantly related paralogues of Ccr4 (Angel, Nocturnin and 2'PDE) and Caf1 (Caf1z) present in higher eukaryotes and it is predicted that there could be further deadenylase enzymes yet to be discovered (Goldstrohm & Wickens, 2008).

mRNA decapping and 5'-3' decay

As mentioned above, once a mRNA has been deadenylated it can be decapped and degraded 5'-3' by Xrn1 (Figure 1.2A) or degraded 3'-5' by the exosome complex (Figure 1.2B). Although there is redundancy between the two pathways, early studies in S. cerevisiae have shown that bulk mRNA decay occurs 5'-3' (Parker, 2012) and studies on higher eukaryotes have further supported the importance of the 5'-3' pathway (Bönisch et al, 2007; Stoecklin et al, 2006), especially those showing this pathway to be essential for microRNA mediated degradation (will be discussed in more detail later) (Izaurralde, 2012).
Figure 1.2. Deadenylation dependent decay.
(Figure on previous page)
Bulk eukaryotic mRNA degradation is deadenylation dependent, which is predominantly carried out by the Ccr4-Not complex. (A) In order for degradation to occur 5'-3' the m’G cap must first be removed. This is achieved by Dcp2, which requires several decapping factors. First, the Lsm1-7/Pat1 complex binds the oligo(A) tract at the 3’ end of the mRNA. This recruits additional decapping factors such as Edc4 and Dcp1 which ultimately activate the decapping reaction by Dcp2. The mRNA is subsequently degraded 5'-3' by Xrn1. (B) Following deadenylation, degradation can also occur 3'-5' by the exosome complex, of which the catalytic domain is Dis3. The m’G cap is then hydrolysed by the scavenger protein DcpS. (C) Deadenylated mRNA can also be uridylated by TUTases which target the mRNA for the exosome independent 3'-5' ribonuclease named Dis3L2.

Pat1/Lsm1-7 complex
A critical step in promoting deadenylation dependent decapping and 5'-3' decay is the displacement of PABP with the Pat1/Lsm1-7 complex (Bouveret et al, 2000; Chowdhury & Tharun, 2009; Tharun et al, 2000; Tharun & Parker, 2001). As mentioned, PABP is bound to the poly(A) tail of translating mRNAs and promotes efficient translation by promoting the circularisation of the mRNP. Once the poly(A) tail is reduced to 10-12 residues, by the Ccr4-Not complex, PABP can no longer bind and instead this small oligo(A) tract becomes a substrate for the Pat1/Lsm1-7 complex (Chowdhury et al, 2007). The binding of this complex to the 3’ end of the mRNA commits the transcript to decapping followed by 5'-3' decay through the recruitment of the decapping machinery and the protection of further 3'-5' decay (He & Parker, 2001; Tharun, 2009; Tharun et al, 2005).

Dcp1/2 complex and decapping activators
The Dcp1/2 complex removes the m’G cap structure from the 5’ end of mRNA. Dcp2 is the catalytic subunit and is a member of the Nudix family of pyrophosphatases (She et al, 2006). There are several decapping factors that stimulate Dcp2 activity, either directly by recruiting Dcp2 to the mRNA or indirectly by repressing translation (Li & Kiledjian, 2010). Dcp1 is essential for efficient decapping by Dcp2 and has been shown in yeast to interact with the N terminal of Dcp2 to promote its catalytic activity, by promoting formation of its closed structure (She et al, 2008; She et al, 2006; She et al, 2004). This interaction is not conserved in higher eukaryotes however, which require the additional co-factor Edc4 (Ge-1), which is not present in yeast (Li & Kiledjian, 2010). Additional decapping factors which enhance decapping directly include Edc1 and Edc2, which are specific to yeast (Dunckley et al, 2001; Schwartz et al, 2003) and Edc3, which is conserved throughout eukaryotes (Kshirsagar & Parker, 2004) and is capable of binding multiple decapping factors, including Dcp1 (Tritschler et al, 2007) and Dcp2 (Harigaya et al, 2010).
Decapping factors which inhibit translation include Scd6 (LSm14a in humans and Tral in *Drosophila*), which directly binds eIF4G (Rajyaguru et al, 2012), the DExD/H box RNA helicase Dhh1 (Rck/p54/DDX6 in humans and Me31B in *Drosophila*) (Coller et al, 2001) which has been shown to coat the mRNA (Ernault-Lange et al, 2012) and inhibit translation elongation (Sweet et al, 2012) and the already mentioned Pat1 (HPat in *Drosophila*) (Coller & Parker, 2005; Marnef & Standart, 2010). Since the cap structure is the substrate for components of both the translational machinery and decapping, it is not surprising that decapping factors act through translational repression. Indeed eIF4E and Dcp2 have been shown to directly compete for the 5’ cap structure (Schwartz & Parker, 2000). Further examples to support this hypothesis include studies showing that when translation initiation is inhibited, deadenylation and decapping increase (Schwartz & Parker, 1999) and that mRNAs which are not efficiently translated, are more rapidly decapped (LaGrandeur & Parker, 1999; Muhrad et al, 1995).

**Nudt16**

Until recently it had long been assumed that Dcp2 was responsible for all cytoplasmic mRNA decapping (Li & Kiledjian, 2010; Ling et al, 2011). However, Song et al. identified an alternative decapping enzyme named Nudt16 in mammalian cells (Song et al, 2010). Nudt16 is also a member of the Nudix family and it was shown that Dcp2 and Nudt16 were able to differentially regulate a subset of genes (Li et al, 2011). Since this discovery, several additional Nudix family proteins have been shown to contain decapping activity *in vitro* (Song et al, 2013) although it remains to be seen whether these are true decapping proteins *in vivo*. It does however seem likely that decapping in higher eukaryotes is more complex than once thought and perhaps similar to deadenylation, contains differentially regulated decapping enzymes, each responsible for distinct roles during development (Geisler & Coller, 2010). Although Nudt16 orthologues have been identified in invertebrate organisms, there is no Nudt16 orthologue in *Drosophila melanogaster*, *Caenorhabditis elegans* or yeast, despite exhaustive searches (Taylor & Peculis, 2008).

**Xrn1**

Once a mRNA is decapped, it is rapidly degraded in a 5’-3’ direction by the exoribonuclease Xrn1. Xrn1 has a well conserved catalytic domain and a less well conserved unstructured domain, thought to be involved in stabilising the catalytic domain as well as binding co-factors such as Dcp1 (Braun et al, 2012). The active site couples unwinding of secondary structure with processivity and can therefore degrade through secondary structures in the absence of a helicase (Parker, 2012).
Xrn1 has been shown to directly bind to the decapping machinery in humans, *Drosophila* and yeast, therefore coupling decapping to 5'-3' degradation. However, the decapping factor to which Xrn1 binds is species specific (Jonas & Izaurralde, 2013). In humans Xrn1 binds Edc4, whereas in *Drosophila* Xrn1 binds the EVH1 domain of Dcp1. Both these interactions occur within the C terminal domain of Xrn1 which is predicted to be predominantly disordered (Braun et al, 2012). In yeast Xrn1 interacts with Pat1 although the mechanism is unknown (Bouveret et al, 2000; Nissan et al, 2010). It is interesting that this interaction between Xrn1 and the decapping machinery is conserved in eukaryotes but that it has been rewired during evolution. Indeed, a conserved property of the decapping machinery seems to be the presence of low complexity disordered regions, in particular short linear motifs, which facilitate protein-protein interactions to allow the formation of decapping complexes (Jonas & Izaurralde, 2013).

### 3'-5' decay

**Dis3 and the exosome complex**

In addition to being decapped and degraded 5'-3' (Figure 1.2A), deadenylated mRNAs can also be degraded 3'-5' by the multi subunit complex named the exosome (Figure 1.2B) (Houseley & Tollervey, 2009; Parker, 2012; Schmid & Jensen, 2008). The exosome consists of a catalytically inert core of nine subunits, consisting of six RNasePH domain proteins (Rrp41/42/43/45/46 and Mtr3) and three RNA binding subunits (Rrp4/40 and Csl4). These proteins form a ring structure similar to the bacterial PNPase (Liu et al, 2006). Enzymatic activity of the complex stems from the tenth subunit, named Rrp44/Dis3 (Dziembowski et al, 2007), which has been shown to have both endo and exoribonuclease activity (Lebreton et al, 2008; Schaeffer et al, 2009; Schneider et al, 2009). The exoribonuclease activity of Dis3 stems from its ribonuclease II like (RNB) domain, whereas the endonuclease activity stems from its PiIT N-terminal (PIN) domain, which is also responsible for its association with the exosome through an interaction with the Rrp41 subunit (Chlebowskiet al, 2013).

In *S. cerevisiae*, this 10 subunit exosome complex localises to both the cytoplasm and the nucleus. It is involved in many different cellular processes such as in the maturation of rRNA, tRNA, snRNA and snoRNA in the nucleus and mRNA degradation in the cytoplasm. Its role is dictated by several cofactors to which it is bound. In order to degrade cytoplasmic mRNAs, the exosome binds the Ski proteins (Ski 7 and the Ski2/3/8 complex). In the nucleus, the exosome is also found bound to Rrp47, Mpp6, an additional 3'-5' exonuclease named Rrp6 and the Tramp complex (Houseley & Tollervey, 2009; Parker, 2012; Schmid & Jensen, 2008).
Dis3-like (Dis3L)

Unlike *S. cerevisiae*, higher eukaryotic organisms possess two additional Dis3 paralogues, named Dis3-like (Dis3L) and Dis3L2. In humans Dis3 was shown to be predominantly localised to the nucleus, whereas Dis3L was shown to be cytoplasmic (Staals et al, 2010; Tomecki et al, 2010). Although both Dis3 and Dis3L contain PIN domains and are found associated with the exosome, only Dis3 was shown to have endonuclease activity (Tomecki et al, 2010).

Dis3L2

Due to its lack of a PIN domain and consequent non-association with the exosome complex, Dis3L2 has only recently been characterised in humans (Lubas et al, 2013), *S. pombe* (Malecki et al, 2013) and plants (named SOV) (Zhang et al, 2010). These studies revealed that Dis3L2 is indeed a cytoplasmic processive 3′-5′ exoribonuclease and as predicted was not found to associate with the exosome complex (Figure 1.2C). Interestingly, Dis3L2 activity was shown to be dramatically increased by the presence of an oligo(U) tract on the 3′ end of its substrate (Chang et al, 2013; Malecki et al, 2013). The importance of this enzyme for mRNA degradation is demonstrated by epistasis experiments carried out in *S. pombe*, which showed mutations in Dis3L2 to be synthetic lethal with mutations in Xrn1 and that mRNA decay is effectively blocked in double mutants for Dis3L2 and LSm1 (Malecki et al, 2013). Furthermore Dis3L2 was shown to associate with Xrn1 in an RNA dependent manner and depletion of Dis3L2 resulted in an increase in P body frequency and the stabilisation of selected mRNAs and a reporter construct containing a well-studied instability element from the TNF-α 3′UTR (Lubas et al, 2013).

In addition to its role in mRNA degradation, Dis3L2 has been implicated in the miRNA biogenesis/degradation pathway where it was shown to be involved in the degradation of *pre-let-7* in mouse embryonic stem cells (Chang et al, 2013; Ustianenko et al, 2013).

1.2.1.2 Deadenylation independent decay

Although it is generally accepted that bulk mRNA decay is deadenylation dependent, there are specific examples of deadenylation independent mRNA decay (Badis et al, 2004; Muhlrad & Parker, 2005). In addition to these specific examples, more widespread mechanisms of deadenylation independent decay also exist in eukaryotic cells.
Uridylation

The view that bulk eukaryotic mRNA degradation was deadenylation dependent was challenged by Rissland and Norbury, who showed that in *S. pombe* decapping and subsequent 5’-3’ decay could be initiated through uridylation, in a mechanism independent of deadenylation (Rissland & Norbury, 2009). In contrast to substrates of Dis3L2, which predominantly undergo deadenylation before the addition of several uridine residues (Gallouzi & Wilusz, 2013; Malecki et al, 2013), mono- or di-uridyl tails are added to mature polyadenylated mRNAs, targeting the mRNA for decapping and 5’-3’ decay. This mono/di-uridylation of the poly(A) tail is thought to target the mRNA to the Lsm1-7 complex. This is in agreement with the observation that the addition of a single uridine to the 3’ end of a mRNA is sufficient to target it to the Lsm1-7 complex *in vitro* (Song & Kiledjian, 2007). This pathway therefore acts in parallel to deadenylation dependent decapping and 5’-3’ decay. In agreement with this, redundancy is observed between the enzyme responsible for this uridylation, named Cid1 (Rissland et al, 2007) and the deadenylase enzyme Ccr4 (Rissland & Norbury, 2009).

It is not clear whether this pathway represents a widespread mechanism for mRNA degradation throughout eukaryotes or whether it is particularly important to *S. pombe*, where poly(A) tail length is shorter than other eukaryotes (Scott & Norbury, 2013). Interestingly *S. cerevisiae* do not contain an orthologue of Cid1 and no uridylation of mRNAs has been reported in this organism. As *S. cerevisiae* has been widely used to characterise mRNA degradation pathways in eukaryotes, this could explain why this mechanism of mRNA decay has only recently been discovered. Humans on the other hand do contain Cid1 homologs, named ZCCHC6, ZCCHC11 and TUT1 (Scott & Norbury, 2013). ZCCHC11 has been shown to be responsible for the uridylation of histone mRNAs (Schmidt et al, 2011). However, these enzymes have not yet been shown to be involved in general turnover of mRNAs in humans.

Uridylation has also been shown to occur on the 5’ cleavage products generated by miRNAs in species as diverse as *Arabidopsis*, mouse and EB virus (Shen & Goodman, 2004). Similar to its effects on polyadenylated mRNAs, this targets the cleavage product to decapping and 5’-3’ decay. It was proposed that the purpose of degrading 5’ cleavage products 5’-3’ rather than 3’-5’ was so that they were not able to be translated into truncated proteins.

Uridylation also appears to play a prominent role in the stability and processing of both pre and mature miRNAs in addition to other small RNAs such as PIWI-interacting RNAs.
(piRNAs), which act synergistically to miRNAs in germ cells to repress transposon activity (Choi et al, 2012; Norbury, 2013; Scott & Norbury, 2013).

**Endonuclease mediated decay**

In addition to undergoing degradation in either the 5'-3' or 3'-5' direction, mRNAs are also subject to endonucleolytic cleavage, which allows exonucleases to attack the unprotected ends of the mRNAs in both directions. Usually the 3' generated fragment is degraded by Xrn1 and the 5' fragment by the exosome (Orban & Izaurralde, 2005), although as mentioned above the 5' fragment can also be degraded by Xrn1 following uridylation (Shen & Goodman, 2004). The most common pathways to utilise endonuclease decay is that of RNA induced silencing complex (RISC) mediated cleavage and during quality control processes such as Nonsense Mediated Decay (NMD). The use of endonuclease mediated cleavage in these processes appears species specific and will be discussed in more detail in the relevant sections of this chapter below. Additionally, as mentioned above, the 3'-5' exoribonuclease Dis3 has been shown to possess endoribonuclease activity through its PIN domain (Lebreton et al, 2008; Schaeffer et al, 2009; Schneider et al, 2009). For an in depth review into endoribonuclease mediated mRNA decay see (Schoenberg, 2011).

**1.2.2 P bodies and Stress granules**

Processing bodies (P bodies) are discrete cytoplasmic foci, containing translationally repressed mRNAs which are either degraded or later returned to translationally competent polysomes (Eulalio et al, 2007a; Franks & Lykke-Andersen, 2008; Kulkarni et al, 2010; Parker & Sheth, 2007). They were first discovered by Bashkirov et al., who noticed the localisation of Xrn1 was highly enriched in discrete prominent foci (Bashkirov et al, 1997). Further studies showed additional components of the 5'-3' degradation pathway and the deadenylase complexes Ccr4-Not and Pan2-Pan3 also co-localised to these cytoplasmic particles (Cougot et al, 2004; Ingelfinger et al, 2002; Temme et al, 2010; van Dijk et al, 2002; Zabolotskaya et al, 2008; Zheng et al, 2008). They were named processing bodies by Roy Parker’s group who showed that they represented sites of 5'-3' degradation (Sheth & Parker, 2003).

In addition to these core proteins, additional P body components have been identified which are only seen in certain species, associated with sub-classes of mRNAs or in certain cell types or conditions. These include components of both the NMD and miRNA pathway, RNA binding proteins and translational repressors as well as proteins affecting viral function (Parker & Sheth, 2007). It had long been believed P bodies only contained proteins
involved in the 5'-3' degradation pathway, as the exosome complex does not localise to P bodies (Sheth & Parker, 2003). However, recent evidence challenges this view in that the exosome independent 3'-5' exoribonuclease Dis3L2 was shown to be associated with P bodies in S. pombe (Malecki et al, 2013).

P body assembly has been shown to be RNA dependent (Teixeira et al, 2005) and is the consequence of RNA mediated repression rather than the cause, as mRNA degradation can occur independently of P body formation (Eulalio et al, 2007b; Stalder & Muehlemann, 2009). The number and size of P bodies is thought to reflect the extent of untranslatable mRNPs available in the cell for aggregation (Cougot et al, 2004; Sheth & Parker, 2003; Zabolotskaya et al, 2008; Zheng et al, 2008), although mRNAs have also been shown to leave P bodies and re-engage with the translational machinery (Bhattacharyya et al, 2006; Brengues et al, 2005). The exact role of P bodies for mRNA decay is therefore still unclear and examples of mRNA decay have even been shown to occur co-translationally (Hu et al, 2009), challenging the view that mRNPs need to disengage with the translational apparatus before undergoing degradation.

Stress granules are also observed as discrete cytoplasmic foci and have substantial overlap in composition and function to P bodies. The distinguishing feature of stress granules as opposed to P bodies is that stress granules consist of mRNPs that have been stalled in the pre-initiation complex of translation and so contain many translation initiation factors, the 40S ribosomal subunit and PABP. Conversely, although Xrn1 is found in stress granules, Dcp1, Dcp2 and GW182 are not. Stress granules form as a result of global translational inhibition caused by different forms of stress, such as heat-shock, oxidative stress, U.V. stress or energy deprivation (Buchan & Parker, 2009; Kedersha et al, 2005).

P bodies and stress granules are also functionally related to mRNP granules observed in neurons (Barbee et al, 2006) and germline cells (Lin et al, 2008), where mRNAs are kept in a translationally repressed state until they are required to undergo translation in a spatio-temporal manner.
1.2.3 Quality control mechanisms

In order to prevent dysfunctional proteins being produced which could have toxic effects to the cell, quality control mechanisms are in place to detect and recognise mRNAs which would exhibit translational defects. These are discussed below.

1.2.3.1 Nonsense Mediated Decay (NMD)

The best studied quality control mechanism is Nonsense Mediated Decay (NMD), which recognises mRNAs containing Premature Termination Codons (PTC) and targets them for destruction. This is important as it prevents the accumulation of potentially harmful truncated proteins. Despite extensive research the mechanism in which this occurs is still controversial and seems to vary between species (Brogna & Wen, 2009; Ghosh & Jacobson, 2010). It is proposed that only newly synthesised mRNAs undergoing a "pioneer" round of translation are susceptible to NMD (Maquat et al, 2010), although this view is widely challenged (Hentze & Izaurralde, 2013). In higher eukaryotes NMD is triggered if a EJC is located at least 50 nucleotides downstream of the stop codon, denoting that codon to be premature (Brogna & Wen, 2009; Ghosh & Jacobson, 2010). However, the EJC is not required for NMD but does facilitate it (Schweigruber et al, 2013). The mechanism more prominent in lower eukaryotes seems to rely on the proximity of the termination codon to its 3' end. If the termination codon is too far away from the 3' end, it is also deemed premature (termed the faux 3'UTR model) (Amrani et al, 2004; Behm-Ansmant et al, 2007).

Although the repertoire of NMD factors varies between species, all eukaryotes possess the conserved set of core NMD factors Upf1, Upf2 and Upf3, which are thought to act as a complex and are essential for NMD in all species tested (Conti & Izaurralde, 2005). In addition to these factors, metazoa also require Smg1, Smg5, Smg6 and often Smg7. This slight difference in the plethora of NMD factors is thought to be behind the different pathways in which NMD is carried out (Conti & Izaurralde, 2005; Nicholson & Muhlemann, 2010). For example, Smg7 is required to promote decapping in mammals, but is not present in Drosophila (Gatfield et al, 2003). Instead, NMD targets undergo endonucleolytic cleavage in Drosophila (Gatfield & Izaurralde, 2004), which is carried out by Smg6 (Huntzinger et al, 2008), which is not present in S. cerevisiae. As mammals possess both Smg6 and Smg7, NMD is proposed to be carried out by both pathways. A putative orthologue of Smg7 has been identified in S. cerevisiae, named Ebs1p (Luke et al, 2007).
In addition to its quality control mechanisms, NMD has also been shown to regulate a subset of wild-type transcripts. The targets regulated by the NMD pathway do not seem to share much functional homology between species (Rehwinkel et al, 2006), suggesting that different organisms have evolved to utilize this mRNA degradation pathway for different purposes. Interestingly, UPF1 null mice are embryonic lethal (Medghalchi et al, 2001), demonstrating the importance of this pathway for normal mammalian development. In addition, the NMD pathway has also been shown to be required for cell proliferation in Drosophila (Rehwinkel et al, 2005b).

### 1.2.3.2 Non Stop Decay (NSD)

mRNAs lacking termination codons undergo Non Stop Decay (NSD) in order to prevent long aberrant mRNAs from being produced and also to release the ribosomes. This requires eukaryotic Release Factor 3 (eRF3) and the exosome co-factor Ski7. It is also thought that translation through the poly(A) tail would displace PABP, leading to deadenylation and subsequent decay (Ghosh & Jacobson, 2010; Isken & Maquat, 2007).

### 1.2.3.3 No Go Decay (NGD)

The process of No Go Decay (NGD) has so far only been studied in *S. cerevisiae*. When translation was blocked by the introduction of a stem-loop structure, the transcript was targeted for endonucleolytic cleavage and subsequent degradation in both directions. This is promoted by the orthologues of eRF3 and eRF1, named Hbs1 and Dom34 respectively (Doma & Parker, 2006).
1.2.4 Regulating mRNA half life

1.2.4.1 ARE (AU-Rich Element) mediated decay

One of the best known cis acting mRNA destabilising elements is the AU-Rich Element (ARE) (Chen & Shyu, 1995). AREs are characterised by the presence of the AUUUA pentamer, surrounded by a U rich context and are located within the 3'UTR of roughly 9% of mRNAs. Interestingly, mRNAs found to contain AREs are frequently associated with processes which require rapid responses, such as inflammation and as such are enriched within mRNAs encoding cytokines, proto-oncogenes and interferons (Anderson, 2008; Ghosh & Jacobson, 2010; Reznik & Andersen, 2010; Schoenberg & Maquat, 2012). AREs are recognised by ARE Binding Proteins (ARE-BPs), such as TTP, BRF1, KSRP and AUF1, which have a destabilising effect on their target and the HuR (ELAV in Drosophila) family of proteins, which have been shown to have a stabilising effect (Ghosh & Jacobson, 2010; Reznik & Andersen, 2010; Schoenberg & Maquat, 2012).

The mechanism in which these ARE-BPs destabilise mRNAs is through the recruitment of the degradation machinery, in which deadenylation is stimulated followed by either 5'-3' or 3'-5' decay, or sometimes both (Figure 1.3) (Chen et al, 2001; Lykke-Andersen & Wagner, 2005; Murray & Schoenberg, 2007; Stoecklin et al, 2006). The exact mechanism by which these proteins recruit the degradation machinery was not clear, but recently TTP has been shown to directly recruit the Ccr4-Not complex through direct binding to Not1 (Sandler et al, 2011). The regulation of ARE-BPs is predominantly through phosphorylation. Under normal conditions they are required to trigger rapid degradation of their targets (Schoenberg & Maquat, 2012).

It is not clear how HuR is able to stabilise mRNAs, although it has been proposed that it can compete for binding of the ARE with the destabilising ARE-BPs (Brennan & Steitz, 2001). A recent genome wide study showed that HuR binds both within introns of pre-mRNA and its 3'UTR and that binding to both provided higher stability than either alone, indicative of HuR in a role in pre-mRNA processing and coupling this to mature mRNA stability (Mukherjee et al, 2011). It was also noticed that HuR bound sites close to micro-RNA binding sites, suggesting a role in antagonising micro-RNA mediated repression (Mukherjee et al, 2011). The regulation of HuR itself is through its shuttling between the cytoplasm and the nucleus (Reznik & Andersen, 2010).
Figure 1.3. ARE mediated decay.

AU rich element binding proteins (ARE BPs) such as TTP bind to AREs located within the 3’UTR of unstable mRNAs such as pro-inflammatory cytokines. This promotes rapid degradation of the mRNA through deadenylation and degradation either 5’-3’ or 3’-5’.
1.2.4.2 microRNAs

microRNAs (miRNAs) are small non-protein coding RNAs of 20-23 nucleotides in length that have emerged as important regulators of post-transcriptional gene regulation in higher eukaryotes (Bartel, 2004; Bartel, 2009; Bushati & Cohen, 2007; Cannell et al, 2008; Jackson & Standart, 2007). Since their discovery in *C. elegans* (Lee et al, 1993; Wightman et al, 1993), the field of miRNA research has exploded and the number of confirmed/predicted miRNAs continues to grow. The latest release of miRBase (v20, June 2013) describes 30,424 mature miRNAs from 206 species, up from 228 miRNAs from five species in the first release of miRBase in 2002 (Kozomara & Griffiths-Jones, 2014). miRNAs have been shown to regulate almost every cellular process studied to date by post-transcriptionally repressing target mRNAs through the recruitment of the miRNA Induced Silencing Complex (miRISC) (Bartel, 2004; Bartel, 2009; Bushati & Cohen, 2007; Cannell et al, 2008; Eulalio et al, 2008; Fabian & Sonenberg, 2012; Fabian et al, 2010; Jackson & Standart, 2007). It is generally accepted that the fate of the mRNA is dependent on the extent of complementarity between the miRNA seed region (nucleotides 2-8) and the mRNA target site (normally within its 3'UTR). High complementarity is associated with cleavage, which appears to be the dominant mechanism in plants, whereas in animals partial complementarity causes translational repression, deadenylation and 5'-3' decay of the mRNA (Izaurrealde, 2012).

Biogenesis

The mechanisms involved in the biogenesis of miRNAs have been extensively reviewed in (Bartel, 2004). To summarise, miRNAs are transcribed as long primary-miRNA (pri-miRNA) precursors, which are cleaved into preliminary-miRNAs (pre-miRNAs) by Drosha and its co-enzyme DGCR8 (Pasha). pre-miRNAs can then be exported into the cytoplasm by Exportin, where they are processed into mature miRNAs by Dicer. pre-miRNAs can also be found in introns and as such are formed during splicing of the mRNA, therefore bypassing the requirement for Drosha. Normally, the passenger strand is degraded and the dominant strand is incorporated into the miRISC by binding Argonaute 1 (Ago1). miRNA biogenesis and degradation has also been shown to be regulated at many levels (Krol et al, 2010; Winter et al, 2009; Yates et al, 2013).

miRISC

The core components of the miRISC are GW182, Ago1 and of course the miRNA itself (Fabian & Sonenberg, 2012). GW182 acts as a scaffold for the complex by binding both Ago1 and then recruiting proteins involved in translational repression and 5'-3' decay. GW182 has been shown to bind both the deadenylase complexes Ccr4-Not and Pan2-Pan3.
(Braun et al., 2011; Chekulaeva et al., 2011; Fabian et al., 2011) causing the release of PABP and therefore disrupting mRNA circularisation (Zekri et al., 2013), as well as recruiting the decapping factors Dcp1, Me31B and Pat1 (Nishihara et al., 2013). Both Ago1 and GW182 are sufficient and required for miRNA mediated silencing (Fabian & Sonenberg, 2012).

**Translational repression or mRNA decay?**

Whether miRNAs repress gene expression by directly inhibiting translation or by degrading the mRNA has been a subject of debate for several years (Cannell et al., 2008; Eulalio et al., 2008; Fabian et al., 2010; Filipowicz et al., 2008; Huntzinger & Izaurralde, 2011; Pillai et al., 2007). It is widely accepted that in plants miRNAs pair with the target mRNA with (almost) perfect complementarity, whereas in metazoans the base pairing is imperfect. It was first proposed that in plants, miRNAs mediate repression by direct cleavage of the mRNA and subsequent degradation, whereas in metazoans, translational repression is the dominant cause of repression with little or no effect on levels of the mRNA (Huntzinger & Izaurralde, 2011). This view has since been challenged as translational repression has been observed in plants (Brodersen et al., 2008; Lanet et al., 2009) and mRNA decay in metazoans (Izaurralde, 2012). As this thesis is focused on gene regulation in animals I will discuss the mechanisms of miRNA mediated repression in metazoans below. For a review on miRNA mediated repression in plants see (Jones-Rhoades et al., 2006).

**miRNA mediated translational repression**

As mentioned, when miRNAs were first discovered, it was observed in *C. elegans* (Olsen & Ambros, 1999; Siggerson et al., 2002) and *Drosophila* (Brennecke et al., 2003) that levels of protein output were repressed, but without any changes to the level of mRNA. The fact that miRNAs were observed in actively translating polysomes suggested that the mechanism of translational repression was post-initiation (Nottrott et al., 2006; Olsen & Ambros, 1999; Petersen et al., 2006; Siggerson et al., 2002).

However, contrasting studies demonstrated that miRNA targeted reporter constructs actually shifted towards the lighter polysome fractions in the presence of its cognate miRNA and that repression of these reporters was dependent on cap mediated translation (Humphreys et al., 2005; Pillai et al., 2005). Since these initial studies there has been increasing evidence to suggest that translational repression induced by miRNAs does indeed occur at the level of initiation (Huntzinger & Izaurralde, 2011).

However, it was not until recently that an actual mechanism of translational repression was proposed. Meijer *et al.* showed that eIF4A2 was required for miRNA mediated repression
and that this was recruited to miRISC through a direct interaction with the Ccr4-Not complex (Meijer et al, 2013). This was perhaps surprising when the role of eIF4A2 had been thought of as being redundant to eIF4A1 and that the two proteins share 90% homology. However, the authors suggest in (Lu et al, 2014) that perhaps the role of eIF4A2 is not to act like a helicase in complex with the eIF4F complex, as is the role of eIF4A1; but rather to act as a clamp on the 5'UTR of miRNA targeted mRNA, similar to the role of the nuclear eIF4A3 in the formation of the EJC (Izaurralde, 2013; Lu et al, 2014).

**miRNA mediated mRNA decay**

Although the above evidence clearly demonstrates an essential role of translational repression in miRNA mediated repression, there is also substantial evidence that mRNA degradation occurs (Baek et al, 2008; Bagga et al, 2005; Guo et al, 2010; Hendrickson et al, 2009; Lim et al, 2005; Selbach et al, 2008) and that this degradation is carried out by the 5'-3' pathway (Behm-Ansmant et al, 2006; Rehwinkel et al, 2005a). Although these studies show that mRNA degradation frequently occurs, they do not demonstrate a requirement for this degradation for translational repression. However, a recent screen using the developing *Drosophila* eye as a model system identified both Dcp1 and Dcp2 as being required for the miRNA mediated repression of two out of three reporter constructs tested (Pressman et al, 2012).

**Temporal order of silencing**

Recent studies have combined the above findings into a temporal order of miRNA silencing, demonstrating that translational repression happens first, followed by deadenylation and subsequent 5'-3' decay (Figure 1.4) (Bethune et al, 2012; Djuranovic et al, 2011; Djuranovic et al, 2012; Hu & Coller, 2012; Izaurralde, 2012).
miRISC is recruited to the mRNA through partial complementarity between the miRNA and the target sequence within the 3’UTR. GW182 then acts as a scaffold to recruit the Ccr4-Not complex (Not2 and Not 3 not shown), which is thought to also recruit elf4A2. elf4A2 then represses translation initiation, possibly by acting as a clamp on the 5’UTR of the mRNA. The mRNA then undergoes deadenylation by the Ccr4-Not complex, followed by decapping and 5’-3’ decay by Xrn1. Based on the models proposed in (Izaurralde, 2012; Izaurralde 2013).
1.2.5 The importance of RNA stability in development and disease

1.2.5.1 Role of mRNA stability in development

mRNA degradation plays an important role in the development of multicellular organisms (Alonso, 2012), particularly during embryonic development, where mRNA degradation rates have been shown to vary dramatically (Thomsen et al, 2010). For example during embryogenesis in *Drosophila*, maternal mRNAs are under translational control to ensure that they are expressed in the correct regions of the embryo where their specific functions are required. These maternal mRNAs are important for restriction of cell fates and almost all undergo specific timed degradation (Cooperstock & Lipshitz, 1997; Johnstone & Lasko, 2001). This is a highly regulated process (Bashirullah et al, 1999; Walser & Lipshitz, 2011) involving both components of maternal origin, such as the RNA binding protein Smaug (Tadros et al, 2007) and components of zygotic origin, such as certain miRNAs (Bushati et al, 2008). Interestingly, piRNAs were recently also shown to be involved in this process (Rouget et al, 2010). The clearance of maternal mRNAs has also been shown to be regulated by miRNAs in zebrafish (Giraldez et al, 2006).

In order for mRNA degradation to act specifically on certain mRNAs at certain times and in certain cell types during development, multicellular organisms have developed a highly complex code relying on *cis* regulatory elements within the mRNA and *trans* acting factors within the cells. The requirement of both these factors to alter the stability of mRNA allows for a highly tissue specific nature of mRNA degradation (Alonso, 2012). In agreement with this, both miRNAs and ARE-BPs show a very tissue specific pattern of expression (Aboobaker et al, 2005; Gouble & Morello, 2000; Lagos-Quintana et al, 2002; Sempere et al, 2003). Indeed miRNAs have been shown to be involved in almost every developmental process studied in *Drosophila* (Jones & Newbury, 2010; Waldron & Newbury, 2012) and it has been postulated that miRNAs confer robustness to developmental programmes (Bushati & Cohen, 2007; Posadas & Carthew, 2014; Stark et al, 2005).

mRNA degradation appears to be most crucial for development during transition periods, such as during the maternal-to-zygotic transition. In agreement with this, mRNAs encoding both the 5'-3' and 3'-5' exoribonucleases, Xrn1 (Pacman) and Dis3 (Tazman), and the exosome component Ski2 (Twister) were shown to be maternally contributed in the embryo and were differentially expressed during *Drosophila* development (Cairrao et al,
2005; Grima et al, 2008; Seago et al, 2001; Till et al, 1998). Furthermore, developmental phenotypes are observed when xrn1 is mutated in Drosophila (Grima et al, 2008; Jones et al, 2013; Zabolotskaya et al, 2008) or knocked down in C. elegans (Newbury & Woollard, 2004), suggesting a role for Xrn1 for the correct development of multi-cellular organisms. The role of Xrn1 (Pacman) in Drosophila development is the focus of this thesis and so will be discussed in more detail below.

1.2.5.2 Diseases associated with RNA stability defects

Since the regulation of mRNA stability during development is highly co-ordinated (Alonso, 2012), it is not surprising that it is of crucial importance for cellular homeostasis and development. mRNA half life is most frequently regulated by cis elements within their 3'UTRs, such as AREs and miRNA binding sites. The importance of ARE mediated regulation for the development of multicellular organisms is best demonstrated by studies using mice to knock out genes encoding ARE-BPs. For example TTP deficient mice display several phenotypes associated with systemic inflammation, attributed to elevated levels of the pro-inflammatory cytokine TNF-α (Sanduja et al, 2011). The importance of regulating mRNA stability through cis elements within 3'UTRs is further highlighted by studies showing that the length of 3'UTRs in cancer cells is frequently shortened (Mayr & Bartel, 2009). Indeed the mis-regulation of miRNAs is strongly associated with cancer development (Nicoloso et al, 2009; Zhang et al, 2007) so much so that they have become attractive candidates for biomarkers of cancer (Bartels & Tsongalis, 2009; Jones et al, 2012a).

There is an increasing number of studies implicating the mis-regulation of exoribonucleases to the development of cancer, especially those involved in the 3'-5' decay pathway (Reis et al, 2013). Mutations in the gene encoding the exosome independent Dis3L2 were shown to be the cause of the Perlman syndrome of overgrowth and susceptibility to Wilms tumour (Astuti et al, 2012). Dis3 has also been identified as a possible tumour suppressor, with recent whole genome wide sequencing analysis identifying mutations in Dis3 associated with multiple myeloma (Chapman et al, 2011; Walker et al, 2012), Acute Myeloid Leukaemia (Ding et al, 2012) and the progression of melanoma (Rose et al, 2011). However, as Dis3 is predominantly localised to the nucleus in humans, it is more likely these diseases are associated with the loss of the nuclear functions of Dis3. In addition, the 5'-3' exoribonuclease Xrn1 was shown to be down-regulated in a number of osteosarcoma cell lines, indicative to a role as a tumour suppressor (Zhang et al, 2002). Further support for the association of the 5'-3' degradation pathway in the development of cancer comes from
studies identifying *Lsm1* and *Dhh1* being overexpressed in pancreatic and colon cancers (Mazzoni & Falcone, 2011).

In addition, viruses have been shown to alter the hosts RNA degradation machinery in order to avoid degradation of their mRNA (Sokoloski et al, 2006). Intricate examples include inhibiting Xrn1 activity through the production of a long non-coding RNA (Moon et al, 2012) and the relocalisation of HuR from the nucleus to the cytoplasm (Barnhart et al, 2013). These examples highlight the importance of RNA stability and also allude to possible therapeutic interventions.
1.3 Advantages of studying RNA stability in *Drosophila melanogaster*

The field of mRNA stability has rapidly developed over the past couple of decades and the role of mRNA degradation in contributing to the regulation of gene expression is now widely accepted (Arraiano et al, 2010; Garneau et al, 2007; Newbury, 2006; Schoenberg & Maquat, 2012). However, despite these advancements, the majority of this work has been carried out in either yeast (Parker, 2012) or immortalized tissue culture cells. Although these systems have enabled the mechanistic aspects of mRNA degradation to be determined, the true significance of mRNA stability during development and disease can only be understood through the use of multi-cellular organisms.

1.3.1 Using *Drosophila melanogaster* as a model organism

*Drosophila melanogaster* is an excellent model system to study the role of mRNA stability in a developmental context as much of the machinery involved in mRNA degradation is highly conserved across eukaryotes (Garneau et al, 2007; Newbury, 2006; Schoenberg & Maquat, 2012). Therefore, studies using *Drosophila* can be directly compared to humans. Although one could argue that using mammalian models such as *M. musculus* would more likely be directly comparable to humans, there are several benefits of using *Drosophila* over mice.

*Drosophila melanogaster* has been used as a model organism since the birth of genetics and as such there is a vast array of genetic tools and resources available. For example, balancer chromosomes allow for both genetic traceability and the retaining of homozygous lethal mutations (Greenspan, 2004). Techniques such as the GAL4/UAS system allow for the expression of any construct in specific spatio-temporal patterns, allowing almost every gene to either be knocked down or overexpressed (Dietzl et al, 2007; Duffy, 2002; Leung & Waddell, 2004). Gene specific mutations can be easily created and genetic screens are more easily accomplished due to the smaller size of the genome (only four chromosomes) and large amount of offspring generated (Greenspan, 2004). Also *Drosophila* husbandry is significantly cheaper and quicker than higher eukaryotes due to their relatively short life cycle.

Research using *Drosophila* as a model organism has made a substantial contribution to many fields in both developmental and cellular biology. For example, in the field of apoptosis, many of the proteins involved in the apoptosis pathway were first discovered in...
Drosophila and the roles of these proteins in a developmental context could be elucidated (Fuchs & Steller, 2011; Hay et al, 2004; Salvesen & Abrams, 2004; Xu et al, 2009). Indeed Drosophila are now an established model organism in which to study diseases such as cancer and its therapeutics (Gonzalez, 2013; Vidal & Cagan, 2006).

1.3.2 Growth and development of Drosophila melanogaster

1.3.2.1 Life cycle of Drosophila melanogaster

The life cycle of Drosophila melanogaster is approximately 8.5 days at 25°C under optimal conditions. Development is slowed with decreasing temperature and is approximately double at 19°C and is faster at increasing temperatures up until 28°C at which point further increased temperature slows development (Ashburner, 1989). The following timings are at 25°C in optimal conditions.

Embryogenesis takes roughly 24h, after which L1 larvae hatch from the egg. There are three larval instars. L1 and L2 last roughly 24h whereas L3 takes 48h and is divided into early and late L3. L1 feed on the surface of the food and only begin to burrow following moulting to L2. The larva continues to feed until late L3, at which point it leaves the food in preparation for metamorphosis (Ashburner, 1989). Larval tissue can be divided into two distinct populations of cells: the larval cells, which are used for larval functions; and imaginal cells, which form discs within the larva and will eventually form all the adult structures of the fly (Ashburner, 1989; Gilbert, 2014b; Wolpert et al, 2011a).

Metamorphosis begins with pupariation, in which the puparium is formed resulting in shortening of the body and hardening of the skin. After 6h the larva retracts from the cuticle in a process termed apolysis which marks the start of pupation. Head eversion occurs roughly 12h after pupariation. During pupation the imaginal discs undergo rapid changes in cell shape and movement and differentiate into the adult cells, whereas the larval tissue is destroyed through programmed cell death. A pharate adult is formed and will eclose as an adult roughly 3.5 days after the onset of pupariation (Ashburner, 1989).

1.3.2.2 Hormonal control of development

The main hormones regulating moulting and metamorphosis in Drosophila are 20-hydroxyecdysone (ecdysone) and Juvenile Hormone (JR). Ecdysone is responsible for initiating the two moult periods and metamorphosis. JH prevents the ecdysone induced
changes in gene expression that occur during metamorphosis, and so JH secretion is inhibited prior to the initiation of metamorphosis (Gilbert, 2014b).

Not surprisingly, the growth of the larva is intimately linked with the onset of metamorphosis. In order for the larva to initiate pupariation it must first reach a critical size. Once this critical size is reached the larva enters a terminal growth period culminating in a peak of ecdysone, the onset of metamorphosis and the cessation of larval growth. During the terminal growth period the larval body can triple in size. The terminal growth period for the imaginal tissue is slightly longer than for the larval tissues as imaginal discs continue to grow until after the initiation of pupation (Shingleton, 2010). Reaching critical size can be delayed by limiting larval growth due to starvation. The link between nutrient availability and hormonal signalling is provided by the insulin/insulin-like growth factor signalling pathway regulated growth of the prothoracic gland (Shingleton, 2010).

In addition to the insulin/insulin-like signalling pathway, prothoraciotropic hormone (PTTH) is also involved in regulating the production of ecdysone. PTTH shows a cyclic profile of expression during the third larval instar and is thought to impose temporal regulation on ecdysone production (McBrayer et al, 2007).

### 1.3.2.3 Patterning of the embryo

During oogenesis, mRNAs are deposited in the egg and the localised expression of these mRNAs is critical in determining both the anterior-posterior (AP) and dorsal-ventral (DV) axis prior to fertilisation (Gilbert, 2014a; Wolpert et al, 2011b).

Following fertilisation, karyokinesis (nuclear division) occurs without cytokinesis (cell division) leading to the formation of a syncytium, in which molecules are able to diffuse freely throughout one continuous cytoplasm. During this time further patterning of the embryo occurs involving a hierarchy of genes further establishing anterior-posterior polarity and dividing the embryo into segments. This is achieved through the establishment of gradients along the AP axis and is reliant on interactions between genes displaying a threshold effect. The first zygotic genes involved in this cascade are the gap genes, followed by the pair-rule genes. The DV boundary in the embryo is formed through the activity of Dorsal, which surprisingly is expressed throughout the syncytial blastoderm. However, only in the ventral region of the embryo does Dorsal enter the nucleus and act as a transcription factor to determine ventral fate (Gilbert, 2014a; Wolpert et al, 2011b).
The majority of these nuclei generated by karyokinesis migrate to the cortex (periphery), while approximately 5 nuclei migrate to the posterior pole of the egg to generate the pole cells which will form the gametes of the adult. The nuclei at the periphery are eventually separated from one another by the cell membrane, creating a cellular blastoderm. This is followed by the mid-blastula transition in which the maternally provided mRNAs are degraded and the control of development is taken over by the zygotic machinery (also termed maternal to zygotic transition). The segment polarity genes are involved in patterning the embryo once cells have formed. Through cell to cell interactions, these genes establish cell fates within the parasegments. Examples of segment polarity genes includes *engrailed*, *wingless* (*wnt*) and *hedgehog*. The pair rule and gap genes also regulate the homeotic selector genes which determine the developmental fate of each segment. For example the fate of the haltere and wing discs is determined by the presence or absence of Ubx expression respectively. Once the embryo becomes cellular, the morphogen Dpp is responsible for determining dorsal fate (Gilbert, 2014a; Wolpert et al, 2011b).

1.3.3 RNA degradation pathways in *Drosophila melanogaster*

The mRNA degradation pathways are highly conserved throughout eukaryotes, with both the mechanisms in which mRNA decay is regulated and the proteins involved showing high conservation between mammals and *Drosophila* (Garneau et al, 2007; Newbury, 2006; Schoenberg & Maquat, 2012). The main difference between humans and *Drosophila* is that humans possess higher diversity in their mRNA degradation machinery, which is not surprising considering the much larger genome and increased complexity.

Bulk mRNA decay is initiated by deadenylation, which is carried out predominantly by the Ccr4-Not complex in both humans and *Drosophila* (Temme et al, 2014). Humans possess two paralogues of both the catalytic components Ccr4 (CNOT6 and CNOT6L) and Caf1 (CNOT7 and CNOT8) compared to one of each encoded by the *Drosophila* genome, which also contains no orthologue of PARN or CAF12 (Goldstrohm & Wickens, 2008). The protein responsible for the deadenylation activity of the Ccr4-Not complex in *Drosophila* appears to be Caf1, as both knockdown and overexpression of a point mutant of Caf1, but not Ccr4, inhibited deadenylation in tissue culture cells (Temme et al, 2010). Knockdown of Caf1 and Not1 but not *Pan2-Pan3* also inhibited miRNA mediated deadenylation of reporter constructs in S2 cells (Behm-Ansmant et al, 2006). Ccr4 may play more of a prominent role in the germ line and during early embryogenesis as null mutants for *Ccr4* (named *twin*) are viable, but display partial female sterility, with embryos deriving from *twin* mutant mothers
showing reduced viability. Flies mutant for Caf1, Not2 and Not3 however are lethal during either embryogenesis or larval stages (Temme et al, 2014).

Decapping in Drosophila is carried out by the decapping protein Dcp2, which is directly stimulated by Dcp1 and Edc4 (Ge-1). Drosophila also possess orthologues of the human decapping factors LSm1-7, Ddx6 (Me31B), LSm14A (Tral), PatL1 (HPat) and Edc3 (Jonas & Izaurralde, 2013). Xrn1 has also recently been shown to act as a decapping factor and directly interacts with Dcp1 in Drosophila (Braun et al, 2012). It is not apparent whether Drosophila possess an orthologue to the mammalian decapping enzyme Nudt16, although orthologues have been found in other invertebrate organisms (Geisler & Coller, 2010; Taylor & Peculis, 2008). The importance in decapping and the 5′-3′ degradation pathway is highlighted by the fact that mutations which inhibit decapping cause lethality to the organism (Fan et al, 2011; Pradhan et al, 2012; Pressman et al, 2012).

Work using Drosophila tissue culture cells suggest that the predominant pathway for mRNA decay is 5′-3′ (Bönisch et al, 2007). Indeed, miRNA mediated degradation of reporter constructs in S2 cells was shown to be dependent on decapping (Behm-Ansmant et al, 2006; Rehwinkel et al, 2005a) with Dcp1, Me31B and HPat directly recruited to miRISC (Nishihara et al, 2013). Interestingly, Dcp1 and Dcp2 were also shown to be required for miRNA mediated repression of two out of three reporter constructs tested in the developing eye of Drosophila. This is the first time that decapping has been shown to be required not only for degradation of the reporter construct but also for translational repression. This does however conflict with evidence demonstrating the requirement of eIF4A2 for miRNA mediated repression (Meijer et al, 2013). However, these experiments were carried out in HeLa cells, which does not mean that the findings will necessarily hold true for other systems. Indeed, Drosophila and C. elegans only have two paralogues of eIF4A; one of which is homologous to the nuclear eIF4A3, and the other which is as homologous to eIF4A1 as it is eIF4A2 (Lu et al, 2014). Although the authors propose that in Drosophila eIF4A1 could function both in translation initiation and in miRNA mediated repression, depending on which protein complexes it is bound to, it is also possible that lower eukaryotes have evolved a unique mechanism for miRNA mediated repression. Indeed, as decapping has been shown to be required for the mRNA decay of certain mRNAs but not others (Eulalio et al, 2007c; Pressman et al, 2012), it is also possible that within humans there are multiple mechanisms with which miRNAs repress gene expression depending on the context of the mRNP. Another possibility which could explain these conflicting results is that mammalian cells have two decapping factors, Dcp2 and Nudt16,
whereas an orthologue of Nudt16 has not been identified in *Drosophila* (Taylor & Peculis, 2008). Indeed, Li *et al.* (2011) showed that these decapping proteins are redundant in miRNA mediated degradation of a reporter construct in MEF cells and only when both proteins are impeded is reporter mRNA degradation alleviated. Therefore decapping may also play an essential role in miRNA mediated repression in human cells, but both Dcp2 and Nudt16 would need to be inhibited for this to be observed (Li *et al.*, 2011). The critical role of miRNAs for *Drosophila* development is demonstrated by the requirement of genes critical for miRNA function for organismal viability (Pressman *et al.*, 2012).

P-bodies are also found in *Drosophila*, containing the 5′-3′ exoribonuclease Xrn1, decapping proteins and also components of the miRNA and NMD machinery (Eulalio *et al.*, 2007a; Zabolotskaya *et al.*, 2008).

Although the above experiments suggest that only the 5′-3′ degradation pathway is required for mRNA decay in *Drosophila*, the exoribonuclease component of the exosome Dis3 has also been shown to be essential for *Drosophila* development (Hou *et al.*, 2012). However, it is not clear whether this is a consequence of its role in the nucleus or cytoplasm. *Drosophila* do not contain an orthologue of Dis3L but do contain the exosome independent 3′-5′ exoribonuclease Dis3L2 which is conserved from *S. pombe* to humans but is absent from *S. cerevisiae* (Lubas *et al.*, 2013; Malecki *et al.*, 2013). Interestingly Dis3L2 has been shown to specifically target uridylated transcripts (Chang *et al.*, 2013; Malecki *et al.*, 2013), however no uridyl transferase has yet been identified in *Drosophila* (Scott & Norbury, 2013). The role of Dis3 and Dis3L2 in *Drosophila* development and the identification of possible uridyl transferases is currently being investigated by Ben Towler in the Newbury lab.

Nonsense mediated decay (NMD) in *Drosophila* is unique compared to other organisms studied in that it is initiated entirely by endonuclease cleavage, followed by decay of the 5′ cleavage product by the exosome and the 3′ fragment by Xrn1 (Gatfield & Izaurralde, 2004). Xrn1 and the exosome are also required to degrade the 3′ and 5′ fragments generated by siRNA directed cleavage (Orban & Izaurralde, 2005). ARE mediated decay is also observed in *Drosophila* (Cairrao *et al.*, 2009), although the polarity of degradation has not yet been shown.

The only cytoplasmic 5′-3′ exoribonuclease identified in eukaryotes is Xrn1, which is highly conserved. Due to the importance of the 5′-3′ degradation pathway for regulated mRNA decay, in both *Drosophila* and throughout eukaryotes, this is an attractive candidate to
investigate the requirement of mRNA stability for the development of multi-cellular organisms. So far only phenotypes of hypomorphic mutations in xrn1/pacman have been extensively studied and characterised in vivo (Grima et al, 2008; Jones et al, 2013; Zabolotskaya et al, 2008). In order to fully understand the function of Pacman in development, it is necessary to analyse the effects of a null mutation to pacman. To this end a new pacman allele has recently been created in the Newbury lab which has been shown, both genetically and molecularly, to be null. Lethality occurs during pupal development, demonstrating that cytoplasmic 5'-3' degradation is essential for viability. The only phenotype observed is that the size of the wing imaginal discs are significantly smaller than wild-type (Jones, 2011). This is particularly interesting as the role of mRNA stability during the growth and development of the wing imaginal discs is not well characterised. Due to the high conservation of the pathways involved in the development of the wing imaginal discs and the large amount of genetic tools available for their study, this is an excellent model system in which to study the role of mRNA stability during organogenesis in multicellular species. The focus of this thesis is therefore to investigate the role of Xrn1/Pacman for the correct growth and development of the wing imaginal discs in Drosophila.
1.4 Using wing imaginal discs to study the regulation of growth and development

The imaginal discs are first specified in the embryo and eventually form all the adult structures of the fly, such as the wings, legs and halteres. Due to the high conservation of signalling pathways and the genetic tools available for their study, the wing imaginal discs have become an ideal model system in which to study the regulation of growth during organ development (Gilbert, 2014b; Wolpert et al, 2011a).

Organ growth and tissue homeostasis are universal processes that occur throughout multicellular organisms and utilise highly conserved signalling pathways such as the morphogens Dpp and Wingless. Interestingly, these morphogens are used in multiple contexts within the development of a single organism, such as during both embryogenesis and imaginal disc development in *Drosophila* (Couso et al, 1993). In addition the regulation of growth by the Hippo signalling pathway is highly conserved between *Drosophila* and mammals and mammalian orthologues of all the components of the pathway exist and can functionally replace their *Drosophila* counterparts (Halder & Johnson, 2011; Neto-Silva et al, 2009). It is therefore likely that many of these mechanisms utilised for the development of the wings in *Drosophila* are utilised during mammalian organogenesis. Using *Drosophila* wing imaginal discs as a model system to study organogenesis has therefore greatly advanced the knowledge and understanding of the cellular mechanisms involved.

1.4.1 Cell fate determination

The wing imaginal discs grow from roughly 50 to 50,000 cells during larval development and eventually form the wing blade and part of the adult thorax (Figure 1.5). During this time the cell fate is determined, but the cells do not differentiate until pupation. Cell fate is determined by a series of complex signalling mechanisms which pattern the disc similar to that used to pattern the embryo. During the first larval instar the disc is first divided into anterior and posterior compartments by the localised expression of Engrailed specifically within the posterior half of the disc. Engrailed is a transcription factor which activates expression of *hedgehog* only in posterior cells. Hedgehog acts as a short range signalling molecule to activate Dpp expression in anterior cells adjacent to the posterior compartment (Zecca et al, 1995). Dpp then diffuses bidirectionally to establish a gradient along the AP axis which specifies cell fate and controls growth of the developing wing imaginal discs. During the second larval instar the disc is further divided into dorsal and
ventral compartments. This is achieved through localized expression of Apterous specifically within dorsal cells (Diaz-Benjumela & Cohen, 1993). Interactions between dorsal and ventral cells leads to expression of the signalling molecule Wingless in a row of cells along the DV boundary (Couso et al, 1994; Diaz-Benjumela & Cohen, 1995; Williams et al, 1993). Wingless then acts as a morphogen by signalling away from the boundary, setting up a gradient along the DV axis. The cells destined to become the wing blade are determined by the expression of Vestigial in the wing pouch of the disc (Couso et al, 1995). The following book chapters provide a more in depth review into the patterning events during wing imaginal disc development (Gilbert, 2014b; Lewis I. Held, 2002; Wolpert et al, 2011a).

1.4.2 Hormonal control of developmental timing

Not surprisingly the growth of the imaginal discs, similar to larval tissue, is intimately linked with developmental timing and the onset of pupariation (Shingleton, 2010). Retarding imaginal disc growth without affecting body size also causes an increase in critical size and a delay in metamorphosis, demonstrating that attainment of critical size is regulated by signals from both larval and imaginal tissues. Interestingly, the signal from the imaginal discs must be negative, as larvae which develop without any imaginal discs are not delayed in metamorphosis (Simpson et al, 1980; Stieper et al, 2008). Therefore, attainment of a "critical state" of the imaginal discs must lead to the inhibition of this negative signal (Shingleton, 2010).
Figure 1.5. Wing imaginal disc fate map.

(A) Photograph of a wild-type wing imaginal disc dissected from a late L3 larva. (B) Fate map of the wing imaginal discs showing the anterior-posterior and dorsal-ventral boundaries. The circle represents the wing pouch which will eventually form the wing whereas the rest of the disc will form the thorax and the wing hinge. (C) Photograph of the dorsal side of a wild-type wing showing the anterior-posterior margin.
1.4.3 Regulation of growth by the Hippo pathway

In addition to determining cell fate, substantial evidence indicates that Dpp and Wingless are required for both the proliferation and survival of the wing imaginal discs cells (Day & Lawrence, 2000). However, growth is evenly distributed throughout the disc (Milan et al, 1996), posing the question of how a morphogen gradient leads to uniform growth. Addressing this issue has long been a challenge for the field and although several mechanisms have been proposed, the subject still remains controversial (Andersen et al, 2013; Baena-Lopez et al, 2012; Day & Lawrence, 2000; Neto-Silva et al, 2009; Schwank & Basler, 2010; Shingleton, 2010).

Recent findings have implicated the highly conserved Hippo pathway in integrating the gradient of Dpp along the AP axis with growth. The Hippo pathway is named after one of two protein kinases in the pathway, named Hippo and Wts, which regulate growth through the phosphorylation of the transcription co-activator Yorkie (Yki). When the pathway is activated, Yki becomes phosphorylated and is unable to enter the nucleus and act as a co-activator. By preventing the phosphorylation of Yki, growth is stimulated through the transcriptional activation of several genes required for growth, survival and proliferation, such as *Myc*, *cyclin E*, *DIAP1* and *bantam*. Loss of Hippo signalling in imaginal discs causes overgrowth, due to increased proliferation and resistance to apoptosis. These discs continue to grow past their normal size and not surprisingly inactivation of Hippo signalling is strongly associated with tumour development (Halder & Johnson, 2011).

The atypical cadherin Fat is a positive regulator of the Hippo pathway and is regulated by Dachsous (Ds). The gradient of Dpp along the AP axis is thought to polarise the expression of Ds and it is proposed that it is the difference in levels of Ds between neighbouring cells that inactivates Fat and therefore prevents the activation of the Hippo pathway, leading to increased growth (Rogulja et al, 2008).

In contradiction to this however, Schwank et al. showed that the role of Fat in regulating the Hippo pathway was not dependent on the gradient of Dpp and instead these two pathways acted in an antagonistic manner to produce uniform growth (Schwank et al, 2011).

The link between the Hippo pathway and Dpp and Wingless is also observed in the nucleus. The transcription factor with which Yorkie binds to promote growth is named Scalloped, which is a downstream effector of the Wingless signalling pathway (Goulev et al, 2008). Furthermore, the downstream effector of Dpp, Mad, was also recently shown to interact
with Yorkie and activate transcription of the anti-apoptotic miRNA bantam (Oh & Irvine, 2011).

1.4.4 Regulation of growth through mechanical force

An alternative model to explain the regulation of growth by Dpp and Wingless integrates mechanisms of physical force. There are two models that have recently been proposed. The first, by Shraiman, suggests that growth is directly induced by Dpp and Wingless, and as such has a stronger effect at the centre of the discs than the periphery. However, this is in direct competition with the effect of compression brought about by increased growth. The increased pressure on cells towards the centre of the disc compared with the periphery therefore antagonises the effect of the morphogen gradient (Shraiman, 2005). The Aegerter-Wilmsen model proposes that the morphogens are only required to promote growth at the centre of the disc as this will induce stretching of the cells at the periphery of the disc, thereby inducing them to undergo further growth to compensate (Aegerter-Wilmsen et al, 2007; Aegerter-Wilmsen et al, 2012).

1.4.5 Cell competition

In order for the imaginal discs to reach a certain size it could be presumed that the cells are programmed to divide a fixed number of times. However, studies have shown that this is not actually the case and that interactions between cells leads to cell competition. Cell competition is a context dependent process in which cells acquire winner and loser fates, with the loser fates undergoing cell death and the winner cells increased proliferation (Johnston, 2009; Levayer & Moreno, 2013). It was first identified by early experiments which utilised a class of mutations, termed Minute, which cause reduced cell division due to impaired protein synthesis. Minute larvae would develop slower than wild-type larvae, but the overall size of the organism was unaffected. However, when clones of Minute and wild-type cells were present in mosaic wing imaginal discs the wild-type cells would out-compete with the Minute cells causing the Minute clones to be either significantly smaller or completely lost from the population (Morata & Ripoll, 1975). Interestingly, when wild-type imaginal discs developed in slow growing Minute larvae, they would have an extra 20h developmental time, yet would stop growing once they reached their normal size (Martin & Morata, 2006). Furthermore, competition was not shown to occur across compartment boundaries (Morata & Ripoll, 1975), and when only the posterior compartment was made Minute the anterior compartment reached its appropriate size before terminating growth,
allowing the posterior compartment to catch up before pupariation (Martin & Morata, 2006). These experiments led to the hypothesis that patterning is involved in outlining the final structure in advance and that the cells divide and compete in order to fill this structure (Day & Lawrence, 2000).

1.4.6 Termination of growth

Equally as important as promoting growth of the discs is the termination of growth once the intended size is reached. As the experiments investigating cell competition demonstrate, the discs have an intrinsic mechanism to sense and terminate their growth as required. Further evidence that imaginal discs possess an intrinsic mechanism to terminate growth comes from studies in which transplanted wing imaginal discs grown in a permissive environment would grow to approximately the same size as in vivo (Bryant & Levinson, 1985). Interestingly, the mechanism in which this is achieved is thought to sense the overall size of the disc, rather than the number of cell divisions, as interfering with the rate of cell division or the size of the cells did not affect the overall size of the discs (Day & Lawrence, 2000).

It is therefore important for the models integrating morphogen gradients with the regulation of uniformed growth to also integrate a mechanism in which growth is effectively terminated. The first model proposed was the Lawrence and Day model in which it was hypothesised that as the disc grew, the gradient of Dpp flattened and as such became ineffective in stimulating further growth (Day & Lawrence, 2000). However recent evidence disputes this model, as it has been shown that the gradient of Dpp activity does not seem to flatten during growth (Hufnagel et al, 2007).

The more recent Shraiman and Aegerter-Wilmsen models of growth discussed above propose alternative mechanisms in which the mechanical forces constrained upon the organ during growth are ultimately responsible for the attainment of the desired size. Shraiman proposes that growth is terminated in the centre of the disc once the effect of compression is stronger than the effect of the morphogens. At the periphery of the disc growth is terminated once the cells are too far away from the organiser to receive any signalling from the morphogens (Hufnagel et al, 2007; Shraiman, 2005). Aegerter-Wilmsen also concludes that growth at the centre is halted by compression. This will therefore also cause growth at the periphery to terminate, as growth at the periphery is stimulated by the stretching caused by growth at the centre (Aegerter-Wilmsen et al, 2007; Aegerter-Wilmsen et al, 2012).
1.5 Background information on the role of Xrn1/Pacman in Drosophila melanogaster development

Xrn1 is the only known cytoplasmic 5’-3’ exoribonuclease in metazoans and orthologues have been identified in S. cerevisiae (Xrn1p), C. elegans (XRN1), Drosophila (Pacman) and humans (XRN1). Xrn1 is a processive exoribonuclease which degrades decapped mRNAs containing a 5’ monophosphate (Jones et al, 2012b; Nagarajan et al, 2013). In addition to its role in degrading mRNA, Xrn1 has also been shown to act as a decapping factor, both in Drosophila and humans (Braun et al, 2012). Xrn1 also degrades uncapped RNA following mRNA cleavage, such as during NMD (Gatfield & Izaurralde, 2004) or RISC targeted decay (Orban & Izaurralde, 2005).

1.5.1 Structure and conservation of Xrn1

Xrn1 is a relatively large protein (184kDa in Drosophila) and determining its structure had proved problematic. However, the structure of Xrn1/Pacman in Drosophila (Jinek et al, 2011) and in the yeast Kluyveromyces lactis (Chang et al, 2011) has recently been determined (Figure 1.6A). In order to crystallise these proteins, a C terminally truncated form of Xrn1 was used which was made catalytically inactive by a single amino acid substitution. These studies provided structural information on the regions C terminal to the active site, as well as confirming the structure of the catalytic domain, which had already been predicted based on the structure of the nuclear Xrn2, which had been determined previously in S. pombe (Xiang et al, 2009).

The catalytic activity of Xrn1 stems from the N terminal of the protein (amino acids 1-673 in Pacman), which is highly conserved across eukaryotes and also with Xrn2. The active site is formed from a CR1 domain, which is surrounded by a CR2 domain (Figure 1.6B). The 5’ phosphate of the RNA is recognised by a basic pocket formed from four highly conserved amino acids (K93, Q97, R100 and R101 in Pacman) (Jinek et al, 2011). Steric hindrance prevents larger 5’ groups from entering the pocket, therefore protecting capped mRNAs from degradation. Directional processivity is achieved through a Brownian ratchet mechanism, which couples substrate binding to processivity, therefore ensuring the whole transcript is degraded (Jinek et al, 2011). Adjacent to the catalytic region is a PAZ/Tudor domain which is thought to play a role in stabilising the catalytic domain, similar to the role of Rai1 in stabilising Xrn2 (Xiang et al, 2009).
Figure 1.6. Structure and conservation of Xrn1.

(A) Crystal structure of Pacman taken from (Nagarajan et al, 2013), based on structure from (Jinek et al, 2011). The catalytic domain (N-terminal) is shown in blue, the C terminal in grey and three nucleotides of decapped RNA are shown in red. (B) Domains of Xrn1 in humans and Drosophila shows high conservation, particularly within the catalytic CR1 and CR2 regions. These regions are also conserved with the nuclear 5’-3’ exoribonuclease Xrn2 (Rat1), which lacks the domains found C terminal of the catalytic regions. The positions of the Dcp1 Binding Motif (DBM) and EDC4 Binding Motif (EBM) found in Drosophila and human Xrn1 are also shown but are not conserved between species. These are found in the regions which are predicted to be unstructured. Amino acid positions are indicated and percentage similarity is shown.
The C terminal region of Pacman (amino acids 800-1140) was shown to arch over the N-terminal region and is composed of a KOW domain, a Winged helix domain and a SH3-like domain (Figure 1.6B) (Jinek et al, 2011). The role of these domains is not clear and the conservation across eukaryotes is less than within the N terminal catalytic domain (Jones et al, 2012b; Nagarajan et al, 2013). However, it is clear that these domains are required for enzyme activity as mutations in these regions interfere with enzyme activity in S. cerevisiae (Page et al, 1998), K. lactis (Chang et al, 2011) and Drosophila (Jones, 2011). Despite this, removal of the nuclear localisation signal from Xrn2 was able to rescue cytoplasmic RNA turnover in Xrn1 deficient cells, despite lacking these domains (Johnson, 1997). However it was not shown whether this rescue was dependent on Rai1.

The Dcp1 binding domain of Pacman was shown to stem from amino acids 1323-1355 (Figure 1.6B) (Braun et al, 2012), which is present within the region of the protein where the structure has yet to be determined and is predicted to be relatively unstructured. It may well be that this unstructured domain also binds additional components of the 5'-3' degradation pathway. The C-terminal domain of Pacman has also been shown to contain a poly-glutamine repeat, which could be involved in binding additional components of the 5'-3' decay pathway (Jones et al, 2012b). Pacman was also shown to interact weakly but reproducibly with Edc4, but no interaction was observed with Dcp2, Edc3, HPat, Me31B, Tral, Lsm1 or Lsm7 (Braun et al, 2012). Hs XRN1 was shown to interact with EDC4 through the 56 most C terminal residues of XRN1 (Braun et al, 2012). Hs XRN1 was also shown to interact weakly with PatL1 (Braun et al, 2012), which had been reported independently elsewhere (Ozgur et al, 2010). It is interesting that these interactions were shown to occur within the unstructured domains of Xrn1, as decapping complexes have been shown to interact with each other through short linear motifs within unstructured regions. This has allowed interactions between these proteins to rapidly evolve, meaning that the individual wiring of this network is not conserved but that the requirement for these proteins to interact with one another is seen across eukaryotes (Jonas & Izaurralde, 2013).
1.5.2 Phenotypes of xrn1 mutants and redundancy between the 5′-3′ and 3′-5′ pathways

xrn1 was first identified and characterised in S. cerevisiae by Audrey Stevens (Stevens, 1980). Further work from the Stevens lab demonstrated that mutations in Xrn1 caused an increased doubling time and led to the accumulation of deadenylated and partially decapped mRNAs (Hsu & Stevens, 1993; Larimer et al, 1992; Larimer & Stevens, 1990). Independently, mutations in Xrn1 were identified through a number of screens and Xrn1 has been implicated in multiple cellular processes, including nuclear fusion (Kim et al, 1990), sporulation (Tishkoff et al, 1991), chromosome stability (Kipling et al, 1991), microtubule function (Interthal et al, 1995), telomere shortening (Liu et al, 1995) and filamentous growth (Kim & Kim, 2002). However, it is unlikely that the disparate phenotypes observed in Xrn1 mutant cells represent multiple cellular functions of Xrn1, rather that Xrn1 is required to regulate the stability of specific mRNAs and that the phenotypic effects seen are indirect (Jones et al, 2012b). This is supported by several lines of evidence. Firstly, the cytoplasmic localisation of Xrn1 would argue against the protein being directly involved in nuclear processes such as chromosome stability and sporulation for example. Secondly, Xrn1 mutant phenotypes can be rescued by targeting the nuclear 5′-3′ exoribonuclease Xrn2 to the cytoplasm (Johnson, 1997), strongly suggesting that these defects are caused by a lack of cytoplasmic 5′-3′ degradation. Furthermore, synthetic lethality screens carried out to identify mutants that in combination with a null Xrn1 allele would be inviable, identified Ski2 and Ski3, which are co-factors of the exosome and are involved in cytoplasmic mRNA degradation (Johnson & Kolodner, 1995) and the eukaryotic translation initiation factor elF4E (Brown et al, 2000). However, no genes involved in the cellular processes described above were identified, suggesting that Xrn1 does not directly function in these pathways, but more likely regulates the stability of specific mRNAs that regulate these processes.

The phenotypic data above demonstrates that although lethality is only observed in S. cerevisiae when mRNA decay is completely abolished, by inhibiting both the 5′-3′ and 3′-5′ pathways (Anderson & Parker, 1998; Johnson & Kolodner, 1995), the stability of certain mRNAs requires Xrn1 in order to be correctly regulated. Interestingly this is also observed in metazoans such as C. elegans and Drosophila, in which Xrn1 knockout or depletion does not completely abolish cell viability, but instead causes developmental defects, indicative of roles in specific developmental processes.
Knockdown of xrn-1 in *C. elegans* results in ventral enclosure defects and embryonic lethality (Newbury & Woollard, 2004). Ventral enclosure is a process analogous to mammalian hind brain closure and wound healing. Interestingly, hypomorphic mutations in *pacman* also result in defects during both dorsal and thorax closure and also wound healing in *Drosophila* (Grima et al, 2008). This therefore indicates that Xrn1 is involved in processes that effect epithelial sheet sealing and therefore could also be involved in hindbrain closure and wound healing in humans. Hypomorphic mutations in *pacman* also resulted in reduced viability, dull wings, crooked legs (Grima et al, 2008) and smaller wings and wing imaginal discs (Jones et al, 2013), suggesting that Pacman could be required for the correct growth and development of the imaginal discs. Pacman was also shown to be required for spermatogenesis, with males containing hypomorphic *pacman* mutations showing reduced male fertility and reduced production of sperm (Zabolotskaya et al, 2008).

### 1.5.3 Expression of Xrn1 during *Drosophila* development

*pacman* mRNA was first shown to be developmentally regulated by Till et al. who showed that *pacman* expression peaked during early embryonic development, most likely due to its maternal contribution (Till et al, 1998). This peak of expression was also seen by ModENCODE temporal expression data (Graveley et al, 2011a), which shows that *pacman* is expressed at all stages of development, with an almost 8 fold difference between the highest (0-2h) and lowest (22h-24) levels of expression. The ModENCODE tissue expression data show that *pacman* expression is highest in the imaginal discs and central nervous system of L3 larvae and also in the testis and ovaries of adult males and females (Graveley et al, 2011b). This could therefore indicate that Pacman is required for the development of these tissues. This fits with the phenotypic data suggesting that Pacman is required for imaginal disc development (Jones et al, 2013), spermatogenesis (Zabolotskaya et al, 2008) and that Pacman was observed in neuronal RNPs (Barbee et al, 2006). Pacman was shown to be localised to P bodies in *Drosophila* testes (Zabolotskaya et al, 2008) and Pacman protein was also shown to more highly expressed in late larval and early pupal stages (Grima et al, 2008).
1.5.4 Work leading up to this project

In order to investigate the role of *pacman* during *Drosophila* development, a series of mutations within the *pacman* gene have been created. The phenotypes of these mutants have been analysed in order to determine which developmental processes Pacman is required for. As mentioned above, the main phenotypes include defects in epithelial closure mechanisms (Grima et al, 2008), smaller wing imaginal discs (Jones et al, 2013), and reduced male fertility (Zabolotskaya et al, 2008). However, these experiments were carried out on hypomorphic *pacman* mutants (pcm<sup>3</sup> and pcm<sup>5</sup>). In order to fully understand the function of Pacman during *Drosophila* development, it is necessary to analyse the phenotypes of a null mutant, which completely lacks functional Pacman protein. A null mutation of *pacman* (pcm<sup>14</sup>) has since been created, which is lethal during the pupal stage and has significantly smaller wing imaginal discs than wild-type (Jones, 2011).

1.6 Aims of this project

The overall aim of this thesis is to determine the role of Pacman during wing imaginal disc development. In order to achieve this genetic and molecular techniques were used to determine why the wing imaginal discs are significantly smaller in *pacman* mutants. The overall hypothesis being tested is that Pacman is required to degrade specific mRNAs, at specific stages of development within the wing imaginal disc cells, in order for their correct growth and development.

Chapter 3

The aims of the experiments carried out in this chapter were to further characterise the phenotypes of the null *pcm<sup>14</sup>* mutant in order to determine why the wing imaginal discs are smaller. This included determining whether the discs are smaller as a direct consequence of a lack of Pacman within the wing imaginal disc cells. Also in order to address the specificity of the phenotype, experiments were carried out which addressed whether other imaginal discs were affected and also whether the whole larvae is smaller and/or developing slower. The developmental patterning of the wing imaginal discs was also analysed to determine whether the discs were delayed in development in addition to growth. Finally the temporal requirement of Pacman was investigated in order to determine at what stages of development Pacman is required for correct wing development to occur.
Chapter 4
The aims of this chapter were to determine the cause of the reduced growth of the wing imaginal discs. Mosaic analysis was carried out to determine whether the wing imaginal disc cells have a cell autonomous requirement for Pacman. Also, immunocytochemistry was used to determine whether there was an increase in apoptosis or a reduction in cell division in the mutant wing imaginal discs.

Chapter 5
The experiments carried out in this chapter were two fold. First of all genetics was used to determine whether apoptosis was directly responsible for the reduced growth and delayed development of the wing imaginal discs. TaqMan q-RT-PCR was then used to follow up these genetic experiments to determine whether the interaction between Pacman and the apoptosis pathway was direct or indirect.

Chapter 6
The aims of this chapter were to complement the results obtained from chapter 5. cRACE was used to determine whether reaper mRNA was specifically required to be degraded 5'-3' or whether the 3'-5' pathway could compensate in the absence of Pacman.
## 2 Materials and Methods

### 2.1 Drosophila stocks

<table>
<thead>
<tr>
<th>Stock</th>
<th>Genotype</th>
<th>Chr. affected</th>
<th>Source</th>
<th>SFN No.</th>
<th>Experiment/Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>$pcm^{14}$</td>
<td>$w^{1118} pcm^{14}$/FM7i P(ActGFP)JMR3</td>
<td>X</td>
<td>P-element excision (Chris Jones)</td>
<td>289</td>
<td>Throughout Null mutant of $pcm$</td>
</tr>
<tr>
<td>$pcm^{5}$</td>
<td>$w^{1118} pcm^{5}$/FM7i P(ActGFP)JMR3</td>
<td>X</td>
<td>P-element excision (Chris Jones)</td>
<td>164</td>
<td>Previous work Hypomorphic mutant of $pcm$</td>
</tr>
<tr>
<td>$pcm^{WT}$</td>
<td>$w^{1118} pcm^{SOE}$</td>
<td>X</td>
<td>P-element excision</td>
<td>295</td>
<td>Throughout Wild-type control for $pcm^{14}$ and $pcm^{5}$</td>
</tr>
<tr>
<td>UAS-$pcm^{WT}$</td>
<td>UAS-$pcm^{38}$</td>
<td>II</td>
<td>Newbury lab (Steve Hebbes)</td>
<td>228</td>
<td>Rescue experiments</td>
</tr>
<tr>
<td>UAS-$pcm^{ND}$</td>
<td>UAS-$pcm^{*21A}$</td>
<td>II</td>
<td>Newbury lab (Steve Hebbes)</td>
<td>255</td>
<td>Rescue experiments</td>
</tr>
<tr>
<td>UAS-$pcm^{RNAi}$</td>
<td>$w^{1118}$, P(GD10926)v21677</td>
<td>III</td>
<td>VDRC No. 21677</td>
<td>283</td>
<td>RNAi knockdown experiments</td>
</tr>
<tr>
<td>69B-GAL4</td>
<td>$w^{*}$, P[w+[mW.hs]=GawB]69B</td>
<td>III</td>
<td>Bloomington No. 1774</td>
<td>394</td>
<td>Wing sizes/ Rescue experiments</td>
</tr>
<tr>
<td>nub-GAL4</td>
<td>P[UAS-Dcr-2.D]1 $w^{1118}$; P(GawB)nubbin-AC-62</td>
<td>X and II</td>
<td>Bloomington No. 25754</td>
<td>387</td>
<td>Wing sizes/ Rescue experiments</td>
</tr>
<tr>
<td>eng-GAL4</td>
<td>eng-GAL4 UAS-GFP/CyO</td>
<td>II</td>
<td>Paul Martin</td>
<td>193</td>
<td>Wing sizes</td>
</tr>
<tr>
<td>Strain Name</td>
<td>Description</td>
<td>Chromosome</td>
<td>Source</td>
<td>Stock Number</td>
<td>Remarks</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
<td>------------</td>
<td>--------</td>
<td>--------------</td>
<td>---------</td>
</tr>
<tr>
<td><strong>act-GAL4</strong></td>
<td>( y^{1} w^{*} ; ) ( P{Act5C-GAL4}2SFO1/ CyO\ y^{+} )</td>
<td>II</td>
<td>Bloomington No. 4414</td>
<td>356</td>
<td>Rescue experiments</td>
</tr>
<tr>
<td><strong>da-GAL4</strong></td>
<td>( da-GAL4 )</td>
<td>III</td>
<td>Barry Denholm</td>
<td>172</td>
<td>Rescue experiments</td>
</tr>
<tr>
<td><strong>arm-GAL4</strong></td>
<td>( w^{*} ; ) ( P{GAL4-arm.S}4a/ TM3, Sb\ y^{1} Ser^{1} )</td>
<td>III</td>
<td>Bloomington No.1561</td>
<td>117</td>
<td>Rescue experiments</td>
</tr>
<tr>
<td><strong>eng-GAL4; GAL80^{ts}</strong></td>
<td>( w^{*}; eng-GAL4/Cyo-GFP; TubGAL80^{ts}/TM6B Tb^{1} )</td>
<td>II and III</td>
<td>Robert Ray</td>
<td>375</td>
<td>GAL80^{ts} wing sizes</td>
</tr>
<tr>
<td><strong>act-GAL4; GAL80^{ts}</strong></td>
<td>( w^{*}; act-GAL4/Cyo-GFP; TubGAL80^{ts}/TM6B Tb^{1} )</td>
<td>II and III</td>
<td>SFN 375 and 356</td>
<td>398</td>
<td>GAL80^{ts} survival</td>
</tr>
<tr>
<td><strong>FRT19A</strong></td>
<td>( y^{1} w^{1118} P{neoFRT}19A )</td>
<td>X</td>
<td>Bloomington No. 1744</td>
<td>361</td>
<td>Mosaic analysis</td>
</tr>
<tr>
<td><strong>FLP FRT19A</strong></td>
<td>( P{Ubi-mRFP.nls}1 w^{*} P{hsFLP}12 P{neoFRT}19A )</td>
<td>X</td>
<td>Bloomington No. 31418</td>
<td>362</td>
<td>Mosaic analysis</td>
</tr>
<tr>
<td><strong>Df(3L)H99</strong></td>
<td>( w^{*}; ) ( Df(3L)H99/ kni^{11} \ p^{0}/ TM6B Tb^{1} )</td>
<td>III</td>
<td>Bloomington No. 1576 (switched balancer)</td>
<td>435</td>
<td>Inhibiting apoptosis</td>
</tr>
<tr>
<td><strong>Ark^{ts}</strong></td>
<td>( y^{1} w^{*} ; ) ( P{lacW}Ark^{11502} \ Ark^{ts} / CyO-GFP )</td>
<td>II</td>
<td>Bloomington No. 23285 (switched Cyo ( y^{+} ) with Cyo-GFP)</td>
<td>447</td>
<td>Inhibiting apoptosis</td>
</tr>
<tr>
<td><strong>UAS-DIAP1</strong></td>
<td>( w^{*}; P{UAS-DIAP1.H}3 )</td>
<td>III</td>
<td>Bloomington No. 6657</td>
<td>424</td>
<td>Inhibiting apoptosis</td>
</tr>
<tr>
<td>Trait</td>
<td>Genotype</td>
<td>Chromosome</td>
<td>Source</td>
<td>Code</td>
<td>Notes</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>-----------------------------------------------</td>
<td>------------</td>
<td>-------------------</td>
<td>------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>rpr-GAL4</td>
<td>( w^*; P{GawB}rpr^{APos20} ) / TM3, Ser(^3)</td>
<td>III</td>
<td>DGRC No. 103634</td>
<td>443</td>
<td>Transcriptional readout of reaper</td>
</tr>
<tr>
<td>White Cambridge</td>
<td>( w^*; Kr^{II-1}/CyO-GFP; MKRS/TM6B Tb(^1)</td>
<td>II and III</td>
<td>Dom Grima</td>
<td>388</td>
<td>Doubly balanced stock used to create stocks/switch balancers</td>
</tr>
</tbody>
</table>
2.2 Drosophila husbandry

2.2.1 Drosophila food recipe

Recipe for 7 litres of fly food:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar (GP)</td>
<td>81g</td>
</tr>
<tr>
<td>Black treacle</td>
<td>410g</td>
</tr>
<tr>
<td>Baker’s yeast</td>
<td>81g</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>40ml</td>
</tr>
<tr>
<td>Oatmeal</td>
<td>616g</td>
</tr>
<tr>
<td>Nipagin</td>
<td>1 spatula</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>7000ml</td>
</tr>
</tbody>
</table>

Fly food was made by Life Sciences technicians and stored at 4°C until required.

2.2.2 Stock maintenance

Stocks used in experiments were kept at 25°C and turned into fresh bottles containing fly food media every two weeks. Back up stocks were kept at 19°C and turned into fresh bottles every 4 weeks.

2.2.3 Cross timings

Stock bottles of flies required for a cross were set up at day 1 (Friday). Adults were tipped off the bottles on day 7 (Friday). Virgin females and males required were collected from day 10-14 (Monday – Friday) and the cross between the collected virgins/males was set up on day 14 (Friday) in bottles or vials. This was repeated for each generation of crosses.

2.2.4 Virgin collection

D. melanogaster females do not mate within the first 8 hours after eclosion (Ashburner, 1989; Greenspan, 2004). The presence of a meconium in the abdomen, visible from the ventral side, ensures flies are only a few hours old and therefore virgins, as it persists for only a few hours after eclosion. For all crosses, flies were selected as virgins only if the meconium was visible. To maximise recovery of virgins, collections were performed twice a day in the morning and afternoon for 5 days (Monday – Friday). Bottles or vials for virgin collection were kept at 19°C overnight and at 25°C during the day.

Virgins were kept in vials at 25°C until the cross was set up. These vials were inspected at least 24h after this to ensure that no larvae were present, which would only occur if non-virgins had been selected.
2.2.5 Staging/selecting larvae

In order to select L3 larvae at 120h After Egg Lay (AEL), crosses were set up on day 1 and L3 wandering larvae were collected 5 days later. They were gently removed from the side of bottles using forceps and placed in 1.5ml tubes prior to use. To select against the GFP present in the FM7i balancer and Cyo-GFP balancer, a Leica MZ16F microscope with a long pass autofluorescence GFP filter was used. Flies containing the balancer FM7i, P{ActGFP}JMR3 (referred to as FM7i in the main text) and CyO-GFP express Actin-GFP which is clearly visible in L3 larvae under UV light, particularly in the gut.

In order to stage larvae to ensure that all larvae were at the same age, short egg lays were carried out. In order for enough eggs to be laid in as short a space of time as possible, at least 10 virgin females and 10 males were used per vial and at least 20 virgin females and 20 males were used per bottle. The cross was set up the previous day to allow flies to mate and acclimatise. The following day the flies were tipped into a fresh vial/bottle and allowed 8h to lay eggs before being removed. The time the flies were removed was recorded as 0h (AEL).

In order to select for larvae just prior to pupariation, bromophenol blue was added to the food (0.05%). This stains the food so that larvae could be selected that had cleared the dyed food from their gut, which occurs just prior to pupariation.

2.2.6 Balancer chromosomes and genetic markers

Balancer chromosomes are commonly used in Drosophila genetics for multiple reasons. Firstly, due to the rearrangements of the chromosomes, they prevent recombination. This allows stocks to be kept through endless generations as the offspring of each generation will be identical. As most balancer chromosomes are homozygous lethal, they are useful when keeping stocks which contain homozygous lethal mutations as they will not take over the stock by selective advantage. Also, as every balancer contains phenotypic markers, the genotypes of the offspring of crosses involving balancer chromosomes are known.

The three main balancer chromosomes used in these experiments are shown in Table 2.2. These were chosen as they all have markers that are identifiable in both the larva and the adult fly.
<table>
<thead>
<tr>
<th>Balancer Chromosome</th>
<th>Insertions</th>
<th>Markers</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>FM7i</td>
<td>P[ActGFP]JMR3</td>
<td>B&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Bar eyes (co-dominant)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>oc&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Female sterility</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ptg&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Darker thoracic trident</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sc&lt;sup&gt;8&lt;/sup&gt;</td>
<td>Loss of scutellar bristle</td>
</tr>
<tr>
<td></td>
<td></td>
<td>w&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Apricot eyes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>y&lt;sup&gt;a3j&lt;/sup&gt;</td>
<td>Yellow body colour (adults)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Brown mouth parts (larvae)</td>
</tr>
<tr>
<td>CyO-GFP</td>
<td>P[GAL4-Hsp70.PB]TR1 P[UAS-GFP.Y]TR1</td>
<td>Cy&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Curly wings</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cn&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Brighter eye colour</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dp&lt;sup&gt;10&lt;/sup&gt;</td>
<td>Thoracic vortex phenotype</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pr&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Reddish-purple eye colour</td>
</tr>
<tr>
<td></td>
<td></td>
<td>nAChRa6&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Insecticide resistance</td>
</tr>
<tr>
<td>TM6B Tb&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
<td>Antp&lt;sup&gt;Hu&lt;/sup&gt;</td>
<td>Extra humeral bristles</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tb&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Larvae and pupae appear shorter</td>
</tr>
<tr>
<td></td>
<td></td>
<td>e&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Darker body colour</td>
</tr>
</tbody>
</table>
2.2.7 L3 imaginal disc dissection for size measurements

Wing imaginal discs were dissected from wondering L3 larvae of the desired genotype in *Drosophila* Ringers solution. First each larva was torn in half and the head end was inverted. Debris was removed to leave the cuticle with the imaginal discs still attached to the larva. The desired imaginal disc was then completely detached from the remaining larval parts. Discs were photographed using a Nikon Digital Sight DS-Fi1 camera mounted on a Nikon SMZ800 dissecting microscope at 5X magnification at 1280X980 pixels. The area of each disc was then measured in arbitrary units (pixels) using ImageJ (rsbweb.nih.gov/ij/) and normalised to wild-type as a percentage. For images, L3 wing imaginal discs were dissected and mounted in Aqua-Poly/Mount (Polysciences, cat. no. 18606-20).

*Drosophila Ringer's solution*: 3mM CaCl$_2$, 182mM KCl, 46mM NaCl, 10mM Tris, adjusted to pH 7.2 with HCl. The solution was autoclaved and passed through a 0.45µm filter prior to use.

2.2.8 L3 wing imaginal disc dissection for RNA extraction and Western Blot

Wing imaginal discs were dissected as above and then transferred to a 1.5ml tube containing 500µl *Drosophila* Ringer’s solution (on ice). This was achieved using a p200 pipette, ensuring no carryover of larval tissue. Wing imaginal discs were collected for 45±5min. The Ringer’s and any floating debris were removed, leaving roughly 20µl Ringer’s and the imaginal discs in the tube. The discs were snap frozen in liquid Nitrogen and stored at -80°C until required.

2.2.9 L3 survival

Larvae of the desired genotype were selected and placed in fresh vials. The number of eclosing adults was counted and this was normalised to wild-type.

2.2.10 Wing measurements

Wings were cut from the body, as close to the notum as possible, using micro-scissors. Once removed the wings were placed in wells of isopropanol in an air tight tissue culture tray for at least an hour to prevent air bubble formation in the wing veins and to preserve the wings until mounting. Wings were then transferred using forceps to a clean microscope slide. To help the transfer a few drops of isopropanol were placed in the middle of the slide to help take the wings off the tips of the forceps. 10-20 wings were placed on each slide.
After the isopropanol had evaporated, 30μl of DPX mounting medium was added onto the wings in a fume hood. A cover slip was slowly then placed on top trying to prevent the formation of any air bubbles. To press down the coverslip and distribute the mounting medium a small brass weight was put on top then after 5 minutes a larger brass weight was positioned on top and the slide was left to set overnight in the fume hood.

A ‘Zeiss Axioplan’ microscope was used to examine the mounted wings. The photos of the wings were taken in colour at 5x magnification using an attached ‘AxioCam MRC Zeiss’ camera. The wings were examined and measured using the Axiovision software. The area of the wing was measured from the alula notch to the base of L5 across to the base of L1, up to the costal break and around the wing margin (Figure 2.1).

2.2.11 Larval weight and size measurements

In order to determine surface area, larvae of the desired genotype were collected before being submerged in ethanol to induce the relaxation of cuticle muscles. This ensured that all individuals were fully extended upon measurement. Once larvae had stopped moving (usually around 10 minutes later), samples were transferred to a black tile and photographed using a Nikon Digital Sight DS-Fi1 camera mounted on a Nikon SMZ800 dissecting microscope. In ImageJ, each image was converted into monochrome so that number of pixels present in the area of the larva could be calculated to give an arbitrary value representing larval size. Photos of an eyepiece graticule were also taken at the magnification used so that these values could then be converted into mm².

In order to weigh the larvae, individual larva were placed in 1.5ml tubes and weighed. The weight of the 1.5ml tube was deducted from this weight to give the weight of the larva.

2.2.12 Larval time measurements

In order to determine larval time, L1 larvae of the desired genotype were collected 24h after a 4h egg lay period, and placed into fresh food vials 10 at a time. These vials were then observed at 9AM and 5PM each day, with any new pupa counted and marked on the outside of the vial in order to ensure they were not mistaken for fresh pupae at a later date.
2.3 Genetic techniques

2.3.1 GAL4/UAS system

The GAL4/UAS system is a commonly used genetic tool in *Drosophila* to express genomic constructs in specific spatio-temporal domains. It can be used to either ectopically express or to knock down genes of interest using RNAi constructs. Furthermore, cDNA constructs can be modified to express tagged versions of proteins, or mutated constructs in which certain domains no longer function as wild-type.

The GAL4/UAS system is bilateral and relies on the yeast gene GAL4 being expressed in certain tissues and then binding to the Upstream Activation Sequence (UAS) present in the UAS construct. There are a wide range of GAL4 stocks available, which each express GAL4 in unique spatio-temporal patterns. Flies containing UAS constructs can then be crossed to these GAL4 flies to drive expression of the construct (Figure 2.2A). This bilateral nature of the system is beneficial as it allows the overexpression/knockdown of essential genes with a one generation cross (Duffy, 2002).

2.3.2 GAL80ts system

The GAL80ts system is an extension of the GAL4/UAS system, in which the GAL80ts provides a mechanism to add temporal control. GAL80ts is a temperature sensitive repressor of GAL4 and is expressed ubiquitously from the *tubulin* promoter. At 19°C the GAL80ts is active and binds the GAL4, inhibiting GAL4 mediated transcription at the UAS. At 29°C however, the GAL80ts becomes inactive and disassociates from GAL4, allowing for GAL4 mediated transcription and expression of the UAS construct (Figure 2.2B) (Leung & Waddell, 2004).
Figure 2.2. Diagrammatic representation of the GAL4/UAS and the GAL80<sub>ts</sub> system.

(A) Flies carrying the GAL4 construct under the control of an endogenous promoter will express GAL4 mRNA (and subsequent protein) in the spatio-temporal domain driven by the promoter. Flies carrying a construct under control of the upstream activation sequence (UAS) for GAL4 protein will not express the construct, unless crossed to flies carrying the GAL4 construct. Flies carrying both the GAL4 driver and UAS construct will express the construct in the specific domain of the GAL4 driver.

(B) GAL80<sub>ts</sub> is a temperature sensitive repressor of GAL4. At 19<sup>°</sup>C GAL80<sub>ts</sub> binds GAL4 and prevents it activating expression of the UAS construct. At 29<sup>°</sup>C however, GAL80<sub>ts</sub> is inactivated, allowing for GAL4 mediated expression of the UAS construct.

Figure 2.1. Area of the wing measured for size.

The dotted red line represents the area of the wing which was measured to determine the size of the wings. This was from the alula notch to the base of the L5 vein across to the base of the L1 vein, up to the costal break and around the wing margin.
2.3.3 Mosaic analysis

2.3.3.1 G418 screen

In order to create the \( w^{1118} \) \( pcm^{14} \) \( P\{neoFRT\}19A/FM7i \) stock, flies were screened for the presence of the neo resistance gene present in the \( P\{neoFRT\}19A \) insertion. This was achieved by adding 200µl of G418 at 25mg/ml to the surface of the food, 24h AEL. G418 (Geneticin) blocks translational elongation, causing lethality. Only flies containing the neo resistance gene will survive to adulthood. To ensure the technique would work as desired, a pilot experiment was carried out with \( pcm^{WT} \) (negative control) and \( P\{neoFRT\}19A \) (positive control) flies. As expected, all \( pcm^{WT} \) flies died yet \( P\{neoFRT\}19A \) flies survived to adulthood.

2.3.3.2 Inducing mitotic recombination

In order to achieve mitotic recombination the FLP had to be activated by heat-shock. This was achieved by place vials containing the larvae in a water bath at 37°C for 1h, either 24h or 48h AEL. Vials were sufficiently submerged so that larvae could not crawl above the surface of the water.
2.4 PCR/sequencing

2.4.1 Quick DNA extraction

Adult flies or larvae were homogenised in 50μl of Squish Buffer per whole fly/larvae in a 1.5ml tube using a disposable pestle. Samples were incubated at 37°C for 30min and then at 95°C for 2min.

**Squish buffer:** 10mM Tris (pH 8.2), 1mM EDTA, 25mM NaCl, 200μg/ml Proteinase K (added immediately prior to use).

2.4.2 PCR primer design

PCR primers were designed using NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Annealing temperatures between 57°-63°C were used. NCBI Primer-BLAST includes automatic BLAST against a specified genome (*D. melanogaster*).

2.4.3 PCR on genomic DNA

1μl of genomic DNA was used in a 25μl reaction using AmpliTaq Gold Buffer (2X), diluted to 1X and primers at a final concentration of 200nM.

The reactions were performed in an Applied Biosystems Veriti (96 well) thermal cycler.

Typical cycling conditions were as follows, with steps 2-4 repeated 35 times.

1) Activation step: 95°C 10min

2) Denaturing step 95°C 30sec

3) Annealing step 55°C 30sec

4) Extension step 72°C 1min/kb of product

5) Extension step 72°C 10min

6) Cooling step 4°C ∞

2.4.4 Agarose gel electrophoresis

Gels were prepared as 1% by dissolving 1.5g Agarose in 150ml 1X TBE. This was achieved by microwaving the solution for 1.5min. GelRed (Cambridge Bioscience) was added before the
gels were allowed to set (10,000X dilution) to allow for visualisation of DNA. The gels were run submerged in 1xTBE. 5µl loading buffer (6X) was added to each 25µl PCR sample prior to loading. 10µl of sample + loading buffer was loaded per well and gels were run for ~60min at 120V (depending on size of product/amount of separation required).

*1xTBE (Tris-Borate-EDTA) buffer:* 90mM Tris, 90mM Boric acid, 2mM EDTA.

*Loading buffer* (for 5ml): 1.75g Ficoll 70 (Sigma), 6mg Bromophenol blue, 5mg Xylene cyanol, 3.1ml 0.2M EDTA, 1.9ml H₂O.

### 2.4.5 Gel extraction of PCR products/DNA sequencing

In order to extract bands of DNA from agarose gels, the MinElute Gel Extraction Kit (Qiagen Cat. No. 28604) was used as to the manufacturers’ instructions. For each sample to be sequenced, 3 bands were cut from the gel and dissolved in 1200µl buffer QG in a 2ml tube. Quality and concentration of the purified DNA was checked on a NanoDrop 1000 spectrophotometer (Thermo Scientific) to ensure they conformed to the requirements before being sent to Eurofins MWG Operon sequencing service. The appropriate sequencing primers at a concentration of 2mM were also sent.

### 2.4.6 Mapping/aligning sequence

Mapping and aligning obtained sequences to the published genome available from FlyBase was achieved using the VectorNTI Advance v.11.

### 2.4.7 Primers used

In order to determine between the wild-type *pacman* allele, the *UAS-pcm<sup>WT</sup>/ND* or whether the *pcm<sup>14</sup>* mutation was present the primers shown in Figure 2.3 were used.
Figure 2.3. Primers used to detect different *pacman* alleles/constructs. 

**A** Diagrammatic representation of the location of the primers used to detect different alleles/constructs of *pacman*. *sfn22/pel3r* primers will only amplify a band if the *pcm*\(^{14}\) mutation is present, as the distance between these primers is too large to amplify a band under these conditions without the deletion in *pcm*\(^{14}\) mutants. *pcm*\(^{5}\) /*pcm*\(^{5}\) \(r\) primers will amplify a band 867bp in size in wild-type cells but will also amplify a band of 803bp in size if the UAS-*pcm*\(^{WT}\) or UAS-*pcm*\(^{ND}\) constructs are present, as these do not contain introns (made from cDNA). *Ex8 f/Ex8 r* primers will amplify a band of 691bp in wild-type cells but will not amplify the UAS-*pcm*\(^{WT}\) or the UAS-*pcm*\(^{ND}\) constructs as these primers are located in the introns. Both the *pcm*\(^{5}\) /*pcm*\(^{5}\) \(r\) and *Ex8 f/Ex8 r* primers will not amplify a band in *pcm*\(^{14}\) cells as these are located within the region deleted in *pcm*\(^{14}\) mutants. **B** Sequences of the primers shown in A.
2.5 Quantitative RT-PCR

2.5.1 RNA extraction

RNA extraction for quantitative-RT-PCR (q-RT-PCR) was performed on 30 wild-type and 90 pcm<sup>14</sup> (due to their decreased size) wing imaginal discs, using a MiRVana miRNA isolation kit (Life Technologies, AM1560). The RNA samples were treated with a DNA-free kit (Life Technologies, AM1906) to degrade all gDNA and had their quantity and quality tested on a NanoDrop 1000 spectrophotometer. Each sample was diluted to the same concentration.

2.5.2 cDNA production

The RNA samples were converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, cat. no. 4368814) with random primers. A "no RT" reaction was performed in parallel as a control to confirm that all genomic DNA had been degraded. Each reaction was carried out in duplicate.

2.5.3 q-PCR

q-PCR was performed on each cDNA replicate and "no RT" in duplicate, using TaqMan Universal PCR Master Mix, No AmpErase UNG (Life Technologies, cat. no. 4324018) and an appropriate TaqMan mRNA/pre-mRNA assay (Life Technologies). Each 20µl qPCR contained between 30-60ng of cDNA. All mRNA TaqMan assays used were pre-designed. For custom pre-mRNA assays, 100nt of sequence of the desired target area was submitted to Life Technologies’ web-based Custom TaqMan Assay Design Tool. A "No Template Control" (NTC) was carried out on each primer/probe assay to ensure no self reactivity.

The reactions were incubated under the following conditions, with steps 2 and 3 repeated 50 times. Fluorescence data was collected at the end of step 3 after each cycle.

1) Activation step 95°C 10min
2) Denaturing step 95°C 15sec
3) Extension step 60°C 30sec
Fold change was calculated based on the mean Ct value for each sample from the 4 technical replicates (2 cDNA replicates X 2 qPCR replicates). Rpl32 (Rp49) was used for normalisation.
2.6 Western blotting

2.6.1 Sample preparation

60 wild-type and 180 pcm14 wing imaginal discs were used per sample. These were homogenised in 50µl 2X loading buffer using a disposable pestle. Alternatively, 1 whole fly or larva was homogenised in 150µl 2X loading buffer per sample. Samples were denatured by heating at 100°C for 7min in a heating block. Samples were spun in a centrifuge at max speed for 5min and the supernatant was taken off and the pellet disposed of. Larval samples were spun again at max speed for 5min and the supernatant was taken off with special attention to avoid the high lipid content. 10µl of fly/larval samples and 20µl of wing imaginal disc samples were loaded onto the gel.

2X loading buffer: (pH 6.8): 250mM Tris, 4% SDS, 10% Glycerol, 0.006% Bromophenol blue, 2% β-mercaptoethanol, 2% Protease cocktail inhibitors. The β-mercaptoethanol and protease cocktail inhibitors were added immediately prior to use.

2.6.2 Gel separation/blotting

Tris Acetate SDS buffer stock solution was used with NuPAGE 7% pre-cast Novex gels (Invitrogen). The gel was run for 1 hour at 150V in an X Cell Surelock Novex Mini Cell tank (Invitrogen). Proteins were transferred to Immobilon P transfer membrane (Millipore) for 1 hour at 100V in Transfer buffer in a Mini Transblot Cell (BioRad). The membrane was washed briefly in PBS-T in a tip box before being blocked in blocking buffer on a rocking platform (protein side up) for 1 hour. The membrane was rinsed briefly in wash buffer then probed for protein using the primary antibody (diluted in wash buffer) on the rocking platform overnight at 4°C. The membrane was subsequently washed 4 times for 10min in wash buffer and then incubated with the secondary antibody (diluted in wash buffer) for 1h at room temperature. The membrane was then washed 4 times for 10min in wash buffer, once for 10min in PBS-T and once for 10min in PBS.

Transfer buffer: 25mM Tris, 190mM Glycine, 20% Methanol.

Blocking buffer (100ml): 1 Sachet PBS, 0.1ml Tween 20, 5g Powdered milk (Marvel), dissolved in 100ml of dH2O.

Wash buffer (500ml): 1 Sachet PBS, 0.5ml Tween 20, 2.5g Powdered milk (Marvel), dissolved in 500ml of dH2O.

1x PBS-Tween (1l): 1 Sachet PBS, 1ml Tween 20, dissolved in 1l of dH2O.
1x PBS (1l): 1 Sachet PBS dissolved in 1l of dH$_2$O.

2.6.3 Developing and quantification

Antibody binding was detected using Amersham ECL detection reagents (GE Healthcare, cat. no. RPN2209). Super RX Medical X-ray Film (Fujifilm) was used to visualise the membrane, which was developed using an Xograph Compact X2 machine. Relative quantification of bands was performed in ImageJ. Tubulin was used as a loading control.

2.6.4 Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Species</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pacman</td>
<td>Newbury lab</td>
<td>Rabbit</td>
<td>1:2000</td>
</tr>
<tr>
<td>Tubulin</td>
<td>Sigma</td>
<td>Mouse</td>
<td>1:2000</td>
</tr>
<tr>
<td>Anti-rabbit</td>
<td>Sigma</td>
<td>-</td>
<td>1:80,000</td>
</tr>
<tr>
<td>Anti-mouse</td>
<td>Sigma</td>
<td>-</td>
<td>1:80,000</td>
</tr>
</tbody>
</table>
2.7 Immunocytochemistry

2.7.1 Slide preparation/fixing the cells

Wing imaginal discs were dissected in PBS and placed on poly-L-lysine treated slides (0.1%) (Sigma). The discs were fixed in 40 μl of 4% paraformaldehyde for 15 min. A cover slip was then placed over the discs and the slides were placed in liquid nitrogen to further permeate the cells. The cover slip was removed and the slides were placed in PBS-T in Coplin jars for a minimum of 10 min.

2.7.2 Staining/washes

Once all slides were prepared, wells were created around the discs with evostick adhesive glue. The discs were then blocked in 200 μl blocking solution for 20 min. The discs were then incubated in primary antibody (diluted in 200 μl blocking buffer) overnight at 4°C in a humidity chamber (used tip box half filled with water). The following day the slides were washed 4 times in PBS-T for 10 min in Coplin jars before being incubated with 200 μl secondary antibody (diluted in blocking buffer) for 2 h at room temperature in the dark. Slides were again washed 4 times for 10 min in PBS-T and once in PBS. If samples were counterstained for DNA they were incubated with DAPI (2 μg/ml) for 15 min before a final wash in PBS for 10 min.

**Blocking buffer:** 0.3% Tween 20, 1% fetal calf serum dissolved in PBS.

2.7.3 Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Species</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-Pacman</td>
<td>Newbury lab (Grima et al. 2008)</td>
<td>Rabbit</td>
<td>1:500</td>
</tr>
<tr>
<td>anti-Cleaved Caspase-3</td>
<td>Cell Signalling (cat no. 9661)</td>
<td>Rabbit</td>
<td>1:400</td>
</tr>
<tr>
<td>(Asp175)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-phosphohistone H3</td>
<td>Cell Signalling (cat. no. 9701)</td>
<td>Rabbit</td>
<td>1:400</td>
</tr>
<tr>
<td>(Ser10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antigen</td>
<td>Source</td>
<td>Species</td>
<td>Dilution</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>---------------------------------------------</td>
<td>---------</td>
<td>----------</td>
</tr>
<tr>
<td>anti-Wingless</td>
<td>Developmental Studies Hybridoma Bank (4D4)</td>
<td>Mouse</td>
<td>1:400</td>
</tr>
<tr>
<td>Cy-3-conjugated monoclonal goat anti-rabbit IgG</td>
<td>Jackson ImmunoResearch (cat. no. 711-165-152)</td>
<td>-</td>
<td>1:400</td>
</tr>
<tr>
<td>Cy-3-conjugated monoclonal Donkey anti-mouse IgG</td>
<td>Jackson ImmunoResearch (cat. no.715-165-151)</td>
<td>-</td>
<td>1:400</td>
</tr>
<tr>
<td>FITC-conjugated polyclonal goat anti-rabbit</td>
<td>Sigma (cat. no. F9887)</td>
<td>-</td>
<td>1:200</td>
</tr>
</tbody>
</table>

### 2.7.4 Confocal microscopy/calculating the mitotic index

Images were taken with a Zeiss Axiovert confocal microscope equipped with a LSM520 Meta. In order to count the number of cells in M phase from the phosphohistone H3 staining, the ImageJ plugin, DeadEasy MitoGlia was used (Forero et al, 2010). All discs were stained and photographed under the same conditions using the standard immunocytochemistry protocol above. All settings were kept as default except the minimum threshold was set to 60. The mitotic index was then calculated for each disc using the following calculation; Mitotic index = No. of cells in M Phase/Area of disc.
2.8 cRACE

Total RNA was extracted from 150 wild-type and 450 pcm<sup>14</sup> wing imaginal discs using the miRVana miRNA isolation kit (Life Technologies, cat. no. AM1560). This produces on average ≥15µg RNA (eluted in 100µl dH<sub>2</sub>O). The RNA samples were treated with a DNA-free kit (Life Technologies, cat. no. AM1906) to degrade all gDNA and quantity and quality was tested using the NanoDrop 1000 spectrophotometer (Thermo Scientific).

RNA-grade solutions and tips were used throughout and surfaces were wiped with RNase Zap wipes (Life Technologies AM9786) before use.

In order to generate the capped pool of RNA, two thirds of the RNA was first treated with SAP and then with TAP as follows.

2.8.1 Shrimp alkaline phosphatase (SAP)

- 67µl RNA @ 150ng/µl (10µg)
- 8µL SAP buffer (10X)
- 5µl SAP (5U) (NEB M0371S)

The reaction was incubated in the PCR machine for 30min at 37°C followed by 65°C for 5min to inactivate the enzyme.

RNA was extracted using the miRVana miRNA isolation kit by adding 400µl lysis buffer and 40µl miRNA homogenate to the reaction and following the protocol supplied by the manufacturer. RNA was eluted in 90µl dH<sub>2</sub>O.

2.8.2 Tobacco acid pyrophosphatase (TAP)

- 90µL RNA from 2.8.1
- 10µl TAP buffer (10X) (In fume hood as contains mercaptoethanol)
- 1.25µl (12.5U) TAP (Epicentre T19100)

Reaction was incubated at 37°C for 1hr in PCR machine.

RNA was extracted using the miRVana miRNA isolation kit by adding 400µl lysis buffer and 40µl miRNA homogenate to the reaction and following the protocol supplied by the manufacturer. 100µl RNA was eluted.

The quantity and quality of the RNA extracted was measured using the NanoDrop 1000 spectrophotometer. This represents the capped pool, and an equal concentration of RNA was diluted to 100µl from the remaining RNA for the decapped pool.
2.8.3 RNA ligation

In order to circularise the RNA the following reaction was set up;

- 100μL RNA from 2.8.2
- 220μL dH₂O
- 40μl T4 RNA ligase buffer (10X)
- 40μl ATP
- 1μl T4 RNA ligase (10U) (NEB M0204S)

Samples were incubated at room temperature overnight. RNA was extracted using the miRVana miRNA isolation kit by adding 400μl phenol/chloroform to the sample and following the manufacturers' instructions from that step onwards. RNA was eluted in 100μl dH₂O.

2.8.4 Reverse Transcription

In order to prepare cDNA the MultiScribe Reverse Transcriptase kit (Life Technologies 4311235) was used. The following reaction was set up;

- 1X RT Buffer
- 1.25U/μl MultiScribe Reverse Transcriptase
- 0.5mM dNTPs mixture
- 5.5 mM MgCl₂
- 0.4U/μl RNase Inhibitor
- 200nM RT primer
- 5μl RNA extracted from 2.8.3
- xμl dH₂O (to make final volume of 20μl)

The reaction was incubated in a PCR machine at 48°C for 30min followed by 95°C for 5min.
2.8.5 PCR

In order to amplify the transcripts the following reaction was set up;

- 1X Taq buffer
- 2U Taq polymerase (Life technologies 18038042)
- 0.8mM dNTPs mixture
- 1.5mM MgCl₂
- 500nM primers (F1/R1)
- 1μL cDNA from 2.8.4
- xμl dH₂O (to make final volume of 50μl)

The reaction was incubated at the following temperatures with steps 2-4 repeated 20-25 times.

1) Activation step  95°C  3min
2) Denaturing step 95°C  45sec
3) Annealing Step  55°C  30sec
4) Extension step  72°C  1.5min
5) Extension step  72°C  10min
6) Cooling step  4°C  ∞

This was repeated, expect using the nested primers (F2/R2) and using one fiftieth of the PCR product.

In order to confirm the amplification, 5μl of this PCR product was run on an agarose gel to confirm the presence of a band.
2.8.6 Cloning

The PCR product was cloned into TOP10 E. coli using the TA Cloning Kit with pCR2.1 vector (Life Technologies K2020-20) following the protocol supplied by the manufacturer and as follows. All materials/solutions were sterile throughout.

2.8.6.1 Ligation

In order to ligate the PCR product into the pCR2.1 vector the following reaction was incubated at 14°C overnight;

- 1µl PCR product from 2.8.5
- 1X ligation buffer
- 2µl pCR2.1 vector (25ng/µl)
- 4U T4 DNA Ligase
- xµl dH2O to make 10µl reaction

2.8.6.2 Transformation

In order to transform the ligated vector into the TOP10 E. coli cells 2µl of the ligation reaction was pipetted into the vial containing the thawed cells and was mixed by pipetting up and down. The vials were incubated on ice for 30min, followed by heat-shock at 42°C for 30sec in a heating block. 250µl S.O.C. medium (Life Technologies 15544-034) was added to the vials and incubated in a water bath at 37°C for 1h. 10-50µl of the cells were spread on LB agar (Sigma 52062) plates containing 100mg/ml Carbenicillin (Sigma C3416) and 20mg/ml X-Gal (Sigma B4252) (dissolved in DMSO). When spreading less than 20µl cells, 20µl S.O.C. medium was added to allow even spreading. Plates were incubated at 37°C overnight and were placed at 4°C for 2h the following morning, to allow for proper colour development before selecting colonies.

2.8.6.3 Inoculation

White colonies were selected and grown in 5ml LB broth (Sigma 51208) with 100mg/ml Carbenicillin, overnight at 37°C in a rocking incubator.

2.8.6.4 Mini preps/sequencing

In order to isolate plasmid DNA the QIAprep Spin Miniprep kit (Qiagen 27106) was used. Quality and quantity of plasmid DNA was measured using the NanoDrop 1000
spectrophotometer before being sent off to Eurofins MWG Operon sequencing service to be sequenced using the M13 Reverse primer.

2.9 Statistical analyses

All statistical analyses were performed in GraphPad Prism 6. All data analysed were compatible with parametric tests. Two-sided two-sample t-tests were used to compare the means of single test groups to single control groups. If multiple comparisons were required, a one-way ANOVA was performed with a post-test to compare the means of each possible pair of samples. A significance level of $p<0.05$ was used throughout unless otherwise stated.
3 Characterising the pcm\textsuperscript{14} mutant phenotypes

3.1 Introduction

In order to investigate the role of Pacman during Drosophila development a reverse genetics approach was used, which involves investigating the phenotypes of pacman mutants in Drosophila to elucidate the cellular role of the protein. Previously in the Newbury lab, several mutant alleles for the pacman gene have been created (Figure 3.1A) and the phenotypes for these mutants are discussed below. This thesis investigates why the wing imaginal discs are reduced in size in pacman mutants. The experiments carried out focus on the null mutant, named pcm\textsuperscript{14}, which has previously been created but not extensively characterised phenotypically (Jones, 2011). Using a null mutant is advantageous over using hypomorphic mutations or RNAi as it is a complete knock-out for the pacman gene. Therefore there will be no functional Pacman protein expressed in pcm\textsuperscript{14} cells. It also has the advantage over using RNAi in that there will be no off target effects, providing there are no additional mutations present in the stock. However, RNAi will also be used to complement these experiments, as using the GAL4/UAS system allows for targeted knockdown in specific spatio-temporal patterns.

This chapter will begin by discussing the description of the pacman alleles, followed by the evidence that the pcm\textsuperscript{14} is a null allele and that there are no additional mutations within the stock. The aims of this chapter will then be discussed followed by the results of the experiments carried out.

3.2 Previous Work

3.2.1 Characterisation of the pacman alleles

In order to determine the function of Pacman in the development of Drosophila, the lab has created a series of mutations in the pacman gene using imprecise excision of the P-element P[EP]EP1526. The most extensively studied of these alleles is pcm\textsuperscript{5} which is a hypomorphic allele. A more recent mutation was created, named pcm\textsuperscript{14}, and this has been shown to be a null allele of pacman (Figure 3.1 A).
Figure 3.1. Diagrammatic representation of the *pacman* alleles.

(A) The genomic region of the *pacman* gene and the alleles *pcm5*, *pcm13* and *pcm14*, created by imprecise excision of P[EP]EP1526. Green boxes represent exons of *pcm*, light blue boxes represent wild-type genomic DNA and thin red lines indicate regions of the genomic DNA that are deleted in each allele. The domains of Pacman protein which would be encoded by these alleles is also shown below. The dotted red line shows the region of the *pacman* gene which the TaqMan primer/probes, used to measure levels of *pacman* mRNA, bind. The dotted blue line show the C terminal region of *pacman* (amino acids 1124-1612) which was used to generate the Pacman antibody. *pcm5* is a hypomorphic allele that consists of a 516bp deletion causing the remainder of the coding region to be out of frame (red box). *pcm13* is also a hypomorphic allele consisting of a 2,222bp deletion extending in both directions from the P-element insertion site. *pcm14* is a 3,501bp deletion extending 3,068bp into the 3' of *pcm*, removing exons 7-11 and part of exon 6, as well as the 5' region of CR43260. (B) Relative *pacman* expression levels in *pcmWT*, *pcm5* and *pcm14* whole larvae, measured by q-RT-PCR. (n=3. Error bars represent SEM.) (C) Western blot of *pcmWT*, *pcm5* and *pcm14* L3 larvae incubated with the Pacman antibody. Tubulin was used as a loading control. The two high molecular weight bands seen in *pcmWT* represent Pacman. These are not seen in *pcm5* or *pcm14* (arrows) and the two bands seen in these samples must be non-specific bands, as the gene region encoding the C terminal domain in which the antibody was raised against is deleted in *pcm14* and partially deleted in *pcm5*. As the lower molecular weight bands are only observed in *pcmWT* and not *pcm5* or *pcm14*, these most likely represent some form of modified cleavage or degradation products of Pacman.
pcm5 is a hypomorphic allele (previously characterised in (Grima et al, 2008; Jones et al, 2013; Zabolotskaya et al, 2008) that consists of a 516bp deletion including exons 8 and 9, causing the remainder of the coding region to be out of frame. mRNA transcribed from this allele has been shown to be down-regulated 3-fold by q-RT-PCR (Figure 3.1 B). Western blots using the Pacman antibody also suggest that any protein translated from this mRNA is also significantly down-regulated (Figure 3.1 C). However this antibody was raised against a 54kD C terminal portion of Pacman (amino acids 1124-1612) which is partially deleted in the pcm5 allele, so immunoreactivity of the Pacman antibody to Pacman protein encoded by the pcm5 allele is likely to be reduced. Indeed molecular evidence suggests that the exoribonuclease activity of Pacman protein encoded by pcm5 mRNA is roughly 66% functional and this will be discussed later in this chapter. Although bands are detected with the Pacman antibody, these must be non-specific, as they are also detected in pcm14 samples in which the entire gene region used to generate the Pacman antibody is deleted.

Since pcm5 mRNA would encode for a large portion of the Pacman protein (amino acids 1-1264 out of a total of 1612) including the catalytic domain (amino acids 1-674) and all the C terminal domains which were determined structurally in (Jinek et al, 2011), any protein that is translated from pcm5 mRNA would most likely still have some exoribonuclease activity. Indeed, the authors of (Jinek et al, 2011) reported that the C terminally truncated form of Pacman (amino acids 1-1141), which was used for determining the structure of Pacman, was catalytically competent. Also, in (Page et al, 1998) deletions which remove the extreme C terminal of Xrn1 but do not remove the SH3-like domain, retain 65% exonuclease activity and can complement the growth defect associated with an Xrn1 null mutant (Page et al, 1998).

pcm5 mutants survive till adulthood. This allele was created and characterised in the lab by Dr. Dominic Grima and was shown to be a hypomorphic allele of pacman.

The pcm14 allele is a 3,501bp deletion extending from the P-element insertion site towards pacman, deleting 3,068bp into the 3’ of the gene, completely removing exons 7-11 and part of exon 6. The 5’ of the neighbouring non-coding RNA CR43260 is also deleted. mRNA from this allele is transcribed and has been shown to be at the same level as wild-type pacman mRNA by q-RT-PCR (Figure 3.1 B). Any protein translated from pcm14 mRNA would include only the first 819 amino acids of wild-type Pacman and so would not contain the Winged helix domain, the SH3-like domain or the Dcp1 binding domain and the KOW domain would also be truncated. A similar sized deletion in S. cerevisiae has been shown to reduce exoribonuclease activity to less than 10% (Page et al, 1998). It is unclear whether
any Pacman protein is translated from this allele as the Pacman antibody was created by expressing a C-terminal portion of Pacman, which would not be coded for by pcm\textsuperscript{14} mRNA.

pcm\textsuperscript{14} mutants die during pupation. This allele was created and characterised in the lab by Dr. Chris Jones. It was shown that this allele was indeed a null mutant for pacman and I will discuss the evidence for this below.

3.2.2 Genetic evidence that pcm\textsuperscript{14} is a null mutant

To ensure that the lethality of the pcm\textsuperscript{14} chromosome was due solely to the deletion at the pacman locus, chromosomal crossover was allowed to occur between the pcm\textsuperscript{14} chromosome (\textit{w\textsuperscript{1118} pcm\textsuperscript{14}}) and a chromosome containing the recessive markers \textit{y\textsuperscript{1} cv\textsuperscript{1} v\textsuperscript{1} f\textsuperscript{1} car\textsuperscript{1}}. Phenotypes of hemizygous males carrying recombinant chromosomes were examined and none were found without the \textit{car\textsuperscript{1}} marker, indicating the lethality of the chromosome stemmed from this region, which contains the \textit{pacman} gene (Figure 3.2A). Additionally, a translocation from \textit{X} to \textit{Y}, \textit{T(1;Y)B92} (contains cytogenetic region 18A5-D1), that includes the pacman locus, was able to rescue the lethality of the pcm\textsuperscript{14} chromosome (\textit{w\textsuperscript{1118} pcm\textsuperscript{14}/T(1;Y)B92} survived to adulthood).

It was demonstrated that the pcm\textsuperscript{14} allele is genetically a null allele as it acts as a deficiency. In combination with the pcm\textsuperscript{5} allele, pcm\textsuperscript{5}/pcm\textsuperscript{14} females have the same phenotype as pcm\textsuperscript{5}/Df(1)JA27 or pcm\textsuperscript{5}/Df(1)(ED7452) females cultured at 19°C (Figure 3.2B). The allelic series showing the severity of phenotypes in Figure 3.2B also demonstrates that pcm\textsuperscript{5} is a hypomorphic allele.
Figure 3.2. Genetic evidence that \textit{pcm}^5 is a hypomorphic mutation and \textit{pcm}^{14} is a null mutation.

(A) Location of the genetic markers used to show that the lethality of the \textit{pcm}^{14} mutation stems from the \textit{pacman} locus. When recombination occurred between the two chromosomes, all hemizygous males in the progeny possessed the \textit{car}^1 marker, which demonstrates that the lethality of the \textit{pcm}^{14} chromosome stems from this locus.  

(B) Allelic series showing the phenotypes of a number of combinations of \textit{pacman} alleles and deficiencies. \textit{pcm}^5/\textit{pcm}^5 flies survive till adult-hood. \textit{pcm}^5/Df(1)ED7452 flies become stuck when eclosing from their pupal cases. Df(1)ED7452 is a 17,963bp deficiency that removes four genes including \textit{pacman}. Similar results are obtained for \textit{pcm}^5/Df(1)JA27 which removes at least 69 genes including \textit{pacman}. The same phenotype is observed in \textit{pcm}^5/\textit{pcm}^{14} flies, showing that \textit{pcm}^{14} is a null allele as it acts as a deficiency. This also shows that \textit{pcm}^5 is hypomorphic, as it has a less severe phenotypes than \textit{pcm}^{14}. \textit{pcm}^{14}/\textit{pcm}^{14} and \textit{pcm}^{14}/Df(1)ED7452 are pupal lethal.
As the deletion in the *pcm*<sup>14</sup> allele also deletes the 5’ region of the neighbouring non-coding RNA *CR43260*, it is possible that the observed phenotypes are caused by this rather than the mutation to the *pacman* gene. The hypomorphic allele, *pcm*<sup>13</sup>, is a 2,222bp deletion extending in both directions from the P-element insertion site, deleting 590bp from the 3’ of *pacman* (including exons 10 and 11), 529bp from the 3’ of *Nat1* and entirely deleting *CR43260* (Figure 3.1A). Despite the additional deletions of *CR43260* and the 3’ of *Nat1*, the phenotypes observed in *pcm*<sup>13</sup> mutants are weaker than those seen in *pcm*<sup>5</sup> mutants, in that they have no dull winged phenotype (personal communication with Chris Jones). Also, stocks of *pcm*<sup>13</sup>/FM7i loose the balancer chromosome, due to the selective advantage of *pcm*<sup>13</sup> homo/hemi-zygotes, which does not occur for the *pcm*<sup>5</sup>/FM7i stock (personal communication with Chris Jones). This therefore shows that the deletion in *CR43260* does not contribute to the *pacman* mutant phenotypes. Further evidence for this will be discussed later in this chapter. This also supports the work in (Braun et al, 2012) that the Dcp1 binding motif is important for the cellular function of Pacman to act as a decapping factor, as the genomic region encoding this motif is deleted in *pcm*<sup>5</sup> but not in *pcm*<sup>13</sup>.

### 3.2.3 Molecular evidence that *pcm*<sup>14</sup> is a null mutation

In order to confirm that the *pcm*<sup>14</sup> allele had no enzymatic activity *in vivo*, a novel assay was performed in whole larvae, utilising the process of nonsense-mediated decay (NMD). NMD in *Drosophila* begins by endonucleolytic cleavage, creating two RNA fragments. It has been shown in cell culture that Pacman is required for degradation of the 3’ fragment, while the 5’ fragment is degraded by the exosome complex (Gatfield & Izaurralde, 2004). This information was used to quantify the severity of the hypomorphic *pcm*<sup>5</sup> allele and the null *pcm*<sup>14</sup> allele at the molecular level.

To carry out this assay, double mutants of *pcm*<sup>14</sup> or *pcm*<sup>5</sup> and an allele of *Alcohol dehydrogenase*, *Adh*<sup>fn6</sup>, which contains a premature termination codon (PTC) and is known to undergo NMD (Brogna, 1999), were created (Figure 3.3A). The levels of the 3’ fragment created by endonucleolytic cleavage of the *Adh*<sup>fn6</sup> transcript in the double mutants were compared with the level of the 3’ fragment in the *Adh*<sup>fn6</sup> single mutant and to the undegraded *Adh* transcript in a wild-type control line with the same genetic background as *pcm*<sup>5</sup> and *pcm*<sup>14</sup>. Whole L3 larvae were used as the *pcm*<sup>14</sup> mutants survive throughout this stage.
Using this assay on wild-type larvae, the level of wild-type Adh mRNA (without the PTC) was measured to be 32-fold higher than in the Adh\textsuperscript{fn6} mutant (with the PTC), showing that this 3' fragment is rapidly degraded in wild-type larvae, as expected (Figure 3.3B). In pcm\textsuperscript{14}; Adh\textsuperscript{fn6} larvae, the level of the 3' fragment was on average 64-fold higher than in pcm\textsuperscript{WT}; Adh\textsuperscript{fn6} larvae, which was not significantly different to the 32-fold increase in wild-type larvae, demonstrating that Pacman is required for degradation of the 3' fragment created by NMD in vivo and is congruent with the genetic data showing that pcm\textsuperscript{14} is a null allele.

In pcm\textsuperscript{5}; Adh\textsuperscript{fn6} larvae, the level of the 3' fragment is significantly higher than that in pcm\textsuperscript{WT}; Adh\textsuperscript{fn6} larvae, by 4-fold. This therefore demonstrates that pcm\textsuperscript{5} is a hypomorphic allele as the levels of the 3' fragment are intermediate between pcm\textsuperscript{WT}; Adh\textsuperscript{fn6} larvae and pcm\textsuperscript{14}; Adh\textsuperscript{fn6} larvae. The relative function of the pcm\textsuperscript{5} allele was estimated based on the ability of the pcm\textsuperscript{5} allele to rescue the defective degradation of the 3' fragment observed in pcm\textsuperscript{14}, Adh\textsuperscript{fn6} larvae. The percentage rescue was calculated by comparing the ΔΔCT values from the q-RT-PCR, rather than fold changes, as this allows for the comparison to be carried out on a linear scale. For pcm\textsuperscript{WT}; Adh\textsuperscript{fn6} larvae the ΔΔCT will be 0 (2\textsuperscript{ΔΔCT}=0 fold change), for pcm\textsuperscript{5}; Adh\textsuperscript{fn6} the ΔΔCT will be 2 (2\textsuperscript{ΔΔCT}=2\textsuperscript{4}=4 fold change) and for pcm\textsuperscript{14}; Adh\textsuperscript{fn6} the ΔΔCT will be 6 (2\textsuperscript{ΔΔCT}=2\textsuperscript{6}=64 fold change). Therefore the rescue is ((6-2)/(6-0)*100)=66.6%, which demonstrates that the catalytic activity of the Pacman protein translated from the pcm\textsuperscript{5} allele is roughly 66.6% functional. This fits in with the previous findings that deletions removing the extreme C terminal of pacman, but not the SH3 domain, reduce the catalytic function to 65% that of wild-type in S. cerevisiae (Page et al, 1998).

Because the 3' fragment of a cleaved mRNA is not capped, this assay measures the exoribonuclease activity of Pacman and not any associated decapping activity. As the Dcp1 binding site is deleted in both pcm\textsuperscript{5} and pcm\textsuperscript{14} alleles it may be that the severity of these alleles is at least partly due to the inability of Pacman protein binding to Dcp1 to enhance decapping. Therefore, although the pcm\textsuperscript{5} allele is estimated to have 66.6% exoribonuclease function, its function as a decapping factor would also be impaired in this mutant. The fact that pcm\textsuperscript{5} mutants survive to adulthood however, shows that this function is not essential. Furthermore it is unclear whether the lack of exoribonuclease activity in pcm\textsuperscript{14} mutants is solely responsible for its lethal phenotype, or whether the lack of Dcp1 binding is also partly responsible.
Together, these results show that $pcm^{14}$ is a null allele, that the lethality in $pcm^{14}$ stems from the $pacman$ locus, and that the deletion in $CR34260$ has no detectable effect. This work was carried out by Dr. Chris Jones in the Newbury lab.
Figure 3.3. Estimation of the relative function of Pacman protein produced from the pacman alleles in vivo.

(A) The Adhfn6 allele of Alcohol dehydrogenase contains a splice site mutation which retains an in frame stop codon in the intron. This causes the Adhfn6 mRNA to undergo NMD, during which the 5’ fragment is degraded by the exosome and the 3’ fragment is degraded by Pacman. (B) To estimate the level of Pacman function in the pcm5 and pcm14 alleles, the level of the fragment degraded by Pacman was compared between lines containing the Adhfn6 allele and either pcm5 or pcm14. (n≥11. p<0.001 for all comparisons unless indicated, ns = not significant. Error bars represent standard error).
3.2.4 Phenotypes of the *pacman* mutants

The main phenotypes observed in the *pcm*\(^5\) mutant flies include defects in both dorsal and thorax closure and wound healing (Grima et al, 2008), smaller wings (84\% the size of wild-type) and wing imaginal discs (82\% the size of wild-type) (Jones et al, 2013), and reduced male fertility (Zabolotskaya et al, 2008). As mentioned, these mutants survive to adulthood.

The phenotypes of the *pcm*\(^{44}\) mutants are, as expected, more severe than the hypomorphic *pcm*\(^5\) mutants, in that hemizygous males and homozygous females die during pupation before any adult structures are formed (100\% penetrance). Furthermore, the wing imaginal discs are 45\% the size of wild-type compared with *pcm*\(^5\) wing imaginal discs being 82\% the size of wild-type (Jones, 2011). Due to the pupal lethality of this mutant, wing size or male fertility cannot be measured.
3.3 Aims

During my PhD. I have chosen to focus my investigations on the wing imaginal disc phenotype of pcm14 mutants. The reasons for this is that this mutant is a null allele and therefore there is no detectable cytoplasmic 5'-3' exoribonuclease activity within these cells. pcm5 is a relatively weak allele, in that it has roughly 88% exoribonuclease activity and this allele has been extensively characterised previously. Although the nature of the pcm14 mutation and its main phenotypes have been characterised, the explanation for the reduced size of the wing imaginal discs has yet to be elucidated. I therefore aim to investigate this growth phenotype further.

The aims of this chapter are to further characterise the phenotypes of the null pcm14 mutant by addressing the following questions

- Is the pupal lethality observed in pcm14 mutants a direct consequence of lack of functional Pacman activity?
- Is the exoribonuclease function of Pacman essential for its activity?
- Is the reduced size of the wing imaginal discs in pcm14 mutant larvae a direct consequence of lack of Pacman expression in the wing imaginal disc cells?
- Are other imaginal discs in the larvae also effected in similar ways to the wing imaginal disc?
- Are the wing imaginal discs smaller simply because the whole larvae is smaller and/or developing slower?
- Are the wing imaginal discs developmentally delayed in addition to their growth being retarded?
- At what stage of development is Pacman activity required, for the correct growth and development of the wing imaginal discs?
3.4 Pacman is expressed ubiquitously throughout the wing imaginal disc

In order to investigate the role of Pacman in the development of the wing imaginal discs, it is important to determine the expression pattern of Pacman protein within the wing imaginal discs. As can be seen from Figure 3.4 Pacman is ubiquitously expressed throughout the wing imaginal discs of L3 larvae.

3.5 Pacman expression is essential for Drosophila development

Although it had been shown that the lethality in pcm\textsuperscript{14} mutants was associated with the locus surrounding the pacman gene and that the deletion of the 5' end of CR43260 was not contributing to the pcm\textsuperscript{14} phenotypes, it had not been shown that pcm\textsuperscript{14} lethality was a direct consequence of a lack of Pacman expression. To address this, the GAL4/UAS system was used to express either wild-type pacman cDNA (UAS-pcm\textsuperscript{WT}) or pacman cDNA in which a single amino acid change abolishes the catalytic activity of Pacman (UAS-pcm\textsuperscript{ND}), in different tissues of the developing larvae. This UAS-pcm\textsuperscript{ND} construct contains a single base mutation which causes the critical glutamate in the conserved magnesium binding site to be mutated to glycine (E177G). Alteration of the homologous residue (E178G) in yeast abolishes 99.9% of exonuclease activity by affecting the binding of the critical Mg\textsuperscript{2+} ion in the active site (Page et al, 1998).

In order to express the UAS-pcm\textsuperscript{WT} construct in pcm\textsuperscript{14} larvae it was first crossed into the pcm\textsuperscript{14} stock to generate the stock y\textsuperscript{i} pcm\textsuperscript{14}/FM7i-GFP; UAS-pcm\textsuperscript{WT}/CyO-GFP (Figure 3.5). Virgin females from these stocks were then crossed to males of different GAL4 drivers and the progeny were observed for any rescued males (Figure 3.6).

The three possible genotypes of males which inherited both the GAL4 driver and the UAS-pcm\textsuperscript{WT} from the cross in Figure 3.6A are shown. These are distinguishable from each other due the presence or absence of the different markers on the X chromosomes inherited from the parents. Any FM7i-GFP/Y males could be identified by the Bar eyed phenotype present in this balancer. These flies would also have yellow bodies as the FM7i-GFP balancer contains the recessive y\textsuperscript{i} marker which gives these flies a yellow body colour phenotype. The y\textsuperscript{i} pcm\textsuperscript{14}/Y males could be distinguished from FM7i-GFP/Y males as these have round shaped eyes. These flies would also have yellow bodies as they also contain the recessive y\textsuperscript{i} marker.
Figure 3.4. Pacman is expressed throughout the wing imaginal disc. (A) pcm\textsuperscript{WT} wing imaginal disc stained with the anti Pacman antibody shows that Pacman is expressed ubiquitously throughout the wing imaginal disc. (B) en-GAL4/UAS-GFP; UAS-pcm\textsuperscript{RNAi}/+ wing imaginal disc stained with the anti-Pacman antibody as a control. GFP expression demonstrates where the UAS-pcm\textsuperscript{RNAi} is being expressed and does not overlap with the signal from the Pacman antibody, demonstrating the specificity of the Pacman antibody.
Figure 3.5. Generation of the \( y^{+} \) pcm14/ FM7i-GFP; UAS-pcmWT/Cyo-GFP and the pcm14/ FM7i-GFP; UAS-pcmND/Cyo-GFP stocks.

(A) Diagrammatic representation of the crosses set up to generate the stock \( y^{+} \) pcm14/ FM7i-GFP; UAS-pcmWT/Cyo-GFP. In order to select \( y^{+} \) pcm14/ FM7i-GFP; Cyo-GFP/+ virgin females from cross F0, the markers heart shaped eyes and Cy wings, were selected for. In order to select FM7i-GFP/Y; UAS-pcmWT/Cyo-GFP males from cross F0', the markers Bar eyes and Cy wings, were selected for. It was not possible to distinguish between flies with UAS-pcmWT/Cyo-GFP or +/-Cyo-GFP from the progeny of cross F1. Therefore crosses in the F2 were set up with one virgin female crossed to one male, and left for 48h to mate and lay eggs. The genotypes were then determined by PCR. Only those crosses where \( y^{+} \) pcm14/FM7i-GFP; UAS-pcmWT/Cyo-GFP was crossed to FM7i-GFP/Y; UAS-pcmWT/Cyo-GFP were used to generate the stable stock. In order to generate the \( y^{+} \) pcm14/FM7i-GFP; UAS-pcmND/Cyo-GFP, exactly the same crosses was carried out, just with the \( w^{+} \) Y; UAS-pcmND males used instead of \( w^{+} \) Y; UAS-pcmWT in cross F0'. (B) PCR was carried out on genomic DNA extracted from the parents of the cross in F2. The pcm5 f_pcm5 r primers were used, which are located in exons 8 and 9 of the pacman gene (See Figure 2.1). This will therefore produce a band for the wild-type endogenous gene and a smaller band for the UAS-pcm constructs, as these are made from pacman cDNA and so will not contain the intron between exons 8 and 9. The virgin female and male used to create each stock therefore have both bands as there is a wild-type pacman gene on the FM7i-GFP balancer chromosome and the UAS-pcmWT or UAS-pcmND construct on chromosome II.
A

\[ y^+ pc^{14}_T, UAS-pc^{WT} \]

FM7i-GFP, CyO-GFP

\[ \text{yellow bodied} \]

Bar eyed

\[ w^+; 69B-Gal4 \]

\[ w^+; 69B-Gal4 \]

\[ w^+; 69B-Gal4 \]

\[ w^+; 69B-Gal4 \]

\[ \text{yellow bodied} \]

\[ \text{no markers} \]

B

1Kb

500bp

1Kb

500bp

C

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Temp (°C)</th>
<th>Survives to adulthood?</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcm^{14}/y</td>
<td>19-29</td>
<td>Yes</td>
</tr>
<tr>
<td>pcm^{14}/y</td>
<td>19-29</td>
<td>No</td>
</tr>
<tr>
<td>pcm^{14}/y; UAS-pcm^{WT}/y; 69B-Gal4/+</td>
<td>19 and 29</td>
<td>Yes</td>
</tr>
<tr>
<td>pcm^{14}/y; UAS-pcm^{WT}/y; 69B-Gal4/+</td>
<td>25</td>
<td>No</td>
</tr>
<tr>
<td>pcm^{14}/y; UAS-pcm^{WT}/y; 69B-Gal4/+</td>
<td>25</td>
<td>Yes</td>
</tr>
<tr>
<td>pcm^{14}/y; UAS-pcm^{WT}/y; nub-Gal4</td>
<td>25</td>
<td>No</td>
</tr>
<tr>
<td>pcm^{14}/y; UAS-pcm^{WT}/y; da-Gal4/+</td>
<td>25</td>
<td>Yes</td>
</tr>
<tr>
<td>pcm^{14}/y; UAS-pcm^{WT}/y; omg-Gal4/+</td>
<td>25</td>
<td>Yes</td>
</tr>
<tr>
<td>pcm^{14}/y; UAS-pcm^{WT}/y; oct-Gal4</td>
<td>25</td>
<td>No</td>
</tr>
<tr>
<td>w^{111}; 69B-Gal4/UAS-pcm^{WT}</td>
<td>25</td>
<td>Yes</td>
</tr>
<tr>
<td>w^{111}; oct-Gal4/+; UAS-pcm^{WT}/y</td>
<td>19-29</td>
<td>No</td>
</tr>
<tr>
<td>w^{111}; UAS-pcm^{WT}/y; 69B-Gal4/+</td>
<td>25</td>
<td>Yes</td>
</tr>
<tr>
<td>w^{111}; oct-Gal4/UAS-pcm^{WT}</td>
<td>25</td>
<td>No</td>
</tr>
<tr>
<td>w^{111}; oct-Gal4/UAS-pcm^{WT}</td>
<td>25</td>
<td>No</td>
</tr>
</tbody>
</table>
Figure 3.6. *pcm*\(^{14}\) pupal lethality can be rescued by expressing *UAS-pcm*\(^{WT}\) in *pcm*\(^{14}\) larvae with different GAL4 drivers.

(Figure on previous page)

(A) Diagrammatic representation of the cross set up to test whether 69B-GAL4 expression of *UAS-pcm\(^{WT}\)* could rescue *pcm\(^{14}\)* pupal lethality. All non-Cy offspring from this cross will inherit both the 69B-GAL4 driver and the *UAS-pcm\(^{WT}\)* construct. The three possible genotypes of non-Cy males from this cross are shown, taking into account that the virgin females could be XXY due to non-disjunction. All three of these genotypes are identifiable from their markers. The presence of round eyed, yellow bodied males in the progeny indicates that *pcm\(^{14}\)* lethality has been rescued. The same cross was set up with different GAL4 drivers and also with \(y^{+}\) *pcm*\(^{14}\)/FM7i-GFP; *UAS-pcm\(^{N0}\)/CyO-GFP

virgin females and the presence of round eyed, yellow bodied males was evidence for rescued lethality. (B) PCR to confirm that the round eyed, yellow bodied males from the above cross did indeed have the genotype \(y^{+}\) *pcm*\(^{14}\)/\(Y;\) *UAS-pcm\(^{WT}\)/+; 69B-GAL4/+ . Amplification of a band with the primers sfn22/pel3r (Figure 2.1) indicate the presence of the *pcm*\(^{14}\) mutation, as without this deletion the primers are too far apart to amplify a band under these conditions. No amplification of a band with the Ex8f/Ex8r primers (Figure 2.1) indicate that no wild-type *pacman* allele is present as these are located in the introns either side of Ex8. Amplification of the smaller band with primers *pcm*\(^{14}\)/*pcm*\(^{r}\) (Figure 2.1) indicates that the *UAS-pcm\(^{WT}\)* construct is present but not the endogenous *pacman* gene. This confirms that round eyed, yellow bodied males from this cross are \(y^{+}\) *pcm*\(^{14}\)/\(Y;\) *UAS-pcm\(^{WT}\)/+; 69B-GAL4/+ .

(C) Table showing the genotypes of those which survive to adulthood and those that die during pupation and at different temperatures.

In addition to these two genotypes, \(w^{+}/Y\) males could also appear in the progeny if non-disjunction is occurring in the \(y^{+}\) *pcm*\(^{14}\)/FM7i-GFP; *UAS-pcm\(^{WT}\)/CyO-GFP stock. Non-disjunction is a rare event in which the chromosomes fail to segregate evenly during meiosis. Although this usually causes lethality to the organism, it can lead to viable XXY females which are fertile and viable X0 males which are sterile (Bridges, 1916). Non-disjunction is known to be more common in *Drosophila* stocks due to inbreeding, which means that once a XXY female is present within the stock, it is difficult to lose. If non-disjunction is occurring in this stock, then \(y^{+}\) *pcm*\(^{14}\)/FM7i-GFP/\(Y;\) *UAS-pcm\(^{WT}\)/CyO-GFP females would be present. As these XXY females would not be phenotypically different from the X0 females in the stock, the crosses set up in Figure 3.6A would contain both X0 and XXY females. These XXY females can pass on their Y chromosome to the males in the progeny, which allows these males to inherit their X chromosome from their father. These males would have the genotype \(w^{+}/Y;\) *UAS-pcm\(^{WT}\)/+ 69B-GAL4/+ and so would also have round eyes but not the \(y^{+}\) marker. It is for this reason that when generating the \(y^{+}\) *pcm*\(^{14}\)/FM7i-GFP; *UAS-pcm\(^{WT}\)/CyO-GFP stock, a *pcm*\(^{14}\) chromosome with the \(y^{+}\) marker was used as opposed to the *w*\(^{1118}\) *pcm*\(^{14}\) chromosome.

The crosses were set up as in Figure 3.6A using different GAL4 drivers. When \(y^{+}\) *pcm*\(^{14}\)/FM7i-GFP; *UAS-pcm\(^{WT}\)/CyO-GFP virgin females were crossed to \(w^{+}/Y;\) 69B-GAL4 males at 25°C, all three genotypes of males mentioned above were observed in the progeny. This demonstrated that expression of wild-type *pacman* with the 69B-GAL4 driver
can rescue $pcm^{14}$ pupal lethality. This is genetic evidence to support the hypothesis that the lethality associated with the $pcm^{14}$ mutation is a direct consequence of lack of Pacman expression within the developing organism.

The fact that $w^{*}/Y$ males were also present shows that non-disjunction was indeed taking place in the $y^{1} pcm^{14}/FM7i-GFP; UAS-pcm^{WT}/CyO-GFP$ stock. To confirm molecularly that the $y^{1} pcm^{14}/Y; UAS-pcm^{WT}/+$; 69B-GAL4/+ males had no endogenous wild-type pacman gene present and just the $UAS-pcm^{WT}$ construct, PCR was performed using DNA extracted from these males (Figure 3.6B).

Further evidence that these rescued males were a direct consequence of the $UAS-pcm^{WT}$ expression comes from the observation that no $y^{1} pcm^{14}/Y; CyO-GFP/+; 69B-GAL4/+$ males were observed in the progeny, yet $FM7i-GFP/Y; CyO-GFP/+; 69B-GAL4/+$ and $w^{*}/Y; CyO-GFP/+; 69B-GAL4/+$ were observed.

The 69B-GAL4 driver is known to drive expression throughout the wing, eye, haltere and leg imaginal discs and in ectodermal tissue during stages 9-17 of embryogenesis (Brand, 1997; Brand & Perrimon, 1993). This result shows that pacman expression in these tissues is required for correct development to occur.

In order to quantify the extent of this rescue, $y^{1} pcm^{14}/Y; UAS-pcm^{WT}/+$; 69B-GAL4/+ and $pcm^{WT}$ L3 larvae were placed in fresh vials and the number of eclosing adults were counted. When normalised to the percentage survival of $pcm^{WT}$, the percentage survival of $y^{1} pcm^{14}/Y; UAS-pcm^{WT}/+$; 69B-GAL4/+ larvae was 100% (Figure 3.7), demonstrating that 69B-GAL4 expression of the $UAS-pcm^{WT}$ rescued the $pcm^{14}$ lethality 100%.
Figure 3.7. 69B-GAL4 driven expression of UAS-pcm<sup>WT</sup> rescues pcm<sup>14</sup> lethality 100% at 25°C.

In order to test the percentage rescue of 69B-GAL4 driven expression of UAS-pcm<sup>WT</sup> in pcm<sup>14</sup> mutants, L3 larvae of the desired genotype were placed in fresh vials and the number of eclosing adults was counted. 100% of y<sup>1</sup> pcm<sup>14</sup>/Y; UAS-pcm<sup>WT</sup>/+; 69B-GAL4/+ survived to adulthood when normalised to pcm<sup>WT</sup>. n=60.
This cross was also carried out at 19°C and 29°C. At 19°C y¹ pcm¹⁴/Y; UAS-pcm\(^{WT}+/+\); 69B-GAL4/+ males were observed in the progeny, yet at 29°C they were not. This shows that this rescue is temperature dependent. It is known that GAL4 drivers generally are more efficient at driving expression at 29°C compared with lower temperatures and it may be that at 29°C there is too much Pacman activity which can also be detrimental to the organism. This is supported by the observation that the act-GAL4 driver also failed to rescue whereas the da-GAL4 and arm-GAL4 drivers did indeed rescue lethality at 25°C. These drivers are all ubiquitous drivers, except that act-GAL4 drives expression at higher levels. It could be argued that act-GAL4 did not rescue because of its expression pattern, but this can be countered by the observation that the knock down of pacman in act-GAL4/UAS-pcm\(^{RNAi}\) causes pupal lethality. Furthermore overexpressing the UAS-pcm\(^{WT}\) in a wild-type background using the act-GAL4 also causes pupal lethality and this was shown to be independent on the exoribonuclease activity of Pacman as w*; act-Gal4/UAS-pcm\(^{ND}\) was also pupal lethal. This supports the evidence from (Braun et al, 2012) that Pacman is able to act as a decapping factor and that overexpression of Pacman is able to inhibit decapping, independent of the exoribonuclease activity. Knockdown of pacman in 69B-GAL4/UAS-pcm\(^{RNAi}\) did not cause pupal lethality despite this expression pattern being critical for correct development to occur. Therefore, low levels of Pacman are clearly sufficient for the survival of the organism. It was observed however that these flies were delayed in development as it took roughly an extra day for these flies to eclose when compared with their parental controls. These results therefore highlight that Pacman is not only required for the viability of the organism but also at the correct amount.

In order to determine whether Pacman was required throughout the imaginal discs in which the 69B-GAL4 driver expresses and/or the embryonic expression of 69B-GAL4 in ectodermal tissue during stages 9-17, y¹ pcm¹⁴/FM7i-GFP; UAS-pcm\(^{WT}/CyO-GFP\) virgin females were crossed to nub-GAL4, which is known to drive expression in a more restricted pattern in the wing, haltere and leg discs. No rescued males were observed in the progeny of this cross which demonstrates a broad requirement of Pacman expression for the correct development of Drosophila.
3.6 The exoribonuclease activity of Pacman is essential for *Drosophila* development

It has recently been shown that Pacman can act as a decapping factor, by binding Dcp1 with its Dcp1 binding domain in the C-terminal region of the protein (Braun et al, 2012). The region of the *pacman* gene which codes for the Dcp1 binding domain is deleted in *pcm*\textsuperscript{14} mutants. Therefore the rescued lethality observed above could, at least in part, be caused by the ability of Pacman to act as a decapping factor in addition to its exoribonuclease activity. This is because if decapping and 5'-3' degradation is impeded in *pcm*\textsuperscript{14} mutants, then this could lead to the accumulation of greater numbers of capped transcripts, which are more likely to be translated, than if just 5'-3' exonuclease activity was impeded. Therefore, rescuing decapping but not 5'-3' exonuclease activity, could also rescue the phenotype of *pcm*\textsuperscript{14} mutants. In order to determine whether the UAS-*pcm*\textsuperscript{WT} rescue was solely dependent on the exoribonuclease activity of Pacman, a similar experiment as above was carried out, except that the UAS-*pcm*\textsuperscript{ND} construct was used, as opposed to the UAS-*pcm*\textsuperscript{WT}.

*y^1 pcm*\textsuperscript{14}/FM7i-GFP; UAS-*pcm*\textsuperscript{ND}/CyO-GFP virgin females were crossed with w^*^/Y;; 69B-GAL4 males. Observation of the progeny from this cross revealed that the UAS-*pcm*\textsuperscript{ND} construct was unable to rescue the *pcm*\textsuperscript{14} lethality. This demonstrates that the exoribonuclease activity of Pacman is essential for the correct development of *Drosophila*. 
3.7 *pcm*¹⁴ wing imaginal discs are significantly smaller than wild-type due to a lack of Pacman expression in the wing imaginal disc cells

The main *pcm*¹⁴ phenotype characterised by Dr. Chris Jones during his PhD. was that the wing imaginal discs were significantly smaller than wild-type. *pcm*¹⁴ wing imaginal discs were on average 45% smaller than their wild-type control, *pcm*<sup>WT</sup> (Jones, 2011). This phenotype is also observed in *pcm⁵*, in that their wing imaginal discs were 82% the size of wild-type, which also correlates with their wings being 84% the size of wild-type (Jones et al, 2013).

From this, one can conclude that mutations to *pacman* cause the wing imaginal discs to be reduced in size. One explanation for this is that Pacman is required for the correct development of the wing imaginal discs, as it is required by wing imaginal disc cells to develop correctly. However, another possible explanation is that this phenotype stems from the lack of Pacman expression throughout the developing larvae, which is causing the whole larvae to fail to develop correctly. It has been shown that the growth and development of the larva and the imaginal discs is highly coordinated (Parker & Shingleton, 2011; Stieper et al, 2008). Therefore, delayed growth and development of the larva could be the cause of the reduced size of the wing imaginal discs, rather than a requirement of the wing imaginal disc cells for Pacman activity. In order to test whether the wing imaginal discs were reduced in size directly because of a lack of expression in the wing imaginal disc cells, Pacman was expressed throughout the wing imaginal discs in *pcm*¹⁴ larvae, in an attempt to rescue this phenotype.

The 69B-GAL4 driver is a relatively ubiquitous driver which drives in larval epidermal tissues (personal communication with Juan Pablo Couso) and has been shown to drive expression throughout the wing imaginal discs (Figure 3.8B and C). Wing imaginal disc size was therefore measured in *y¹ pcm*¹⁴/Y; *UAS-pcm*<sup>WT</sup>/+; 69B-GAL4/+ L3 larvae. In order to generate these larvae, the same cross from Figure 3.6A was set up, except virgin females which were homozygous for the *UAS-pcm*<sup>WT</sup> chromosome were used to increase the percentage of desired offspring in the progeny (Figure 3.9A). Non-GFP larvae with brown mouth parts were selected from the progeny as these larvae will all have the desired genotype of *y¹ pcm*¹⁴/Y; *UAS-pcm*<sup>WT</sup>/+; 69B-GAL4+. The wing imaginal discs from these larvae were then dissected and measured.
Figure 3.8. Diagrammatic representation of the expression patterns for the GAL4 drivers used.

(A) Diagrammatic representation of a wing imaginal disc fate map forming the adult wing.
(B) The 69B-GAL4 driver expresses throughout the wing imaginal disc, as seen by the GFP expression in UAS-GFP/+; 69B-GAL4/+ wing imaginal discs (C).
(D) The nub-GAL4 driver expresses throughout the wing pouch of the disc, as seen by the GFP expression in UAS-GFP/nub-GAL4 wing imaginal discs (E).
(F) The en-GAL4 driver expresses throughout the posterior compartment of the wing imaginal disc, as seen by the GFP expression in UAS-GFP/en-GAL4 wing imaginal discs (G).
Figure 3.9D shows that the mean wing imaginal disc size of $y^1 pcm^{14}/Y$; $UAS-pcm^{WT}/+$; 69B-GAL4/+ L3 larvae is not significantly different to wild-type, demonstrating that wild-type Pacman expression throughout the wing imaginal discs of $pcm^{14}$ larvae rescues the wing imaginal disc phenotype. To ensure that the $UAS-pcm^{WT}$ construct was not being expressed ubiquitously throughout the larva in the absence of the 69B-GAL4 driver by “leaky expression”, wing imaginal disc size was also measured in $y^1 pcm^{14}/Y$; $UAS-pcm^{WT}$ L3 larvae and this was not significantly different to the wing imaginal disc size in $pcm^{14}$ L3 larvae.

This supports the hypothesis that the explanation for $pcm^{14}$ mutants having smaller wing imaginal discs is due to a lack of Pacman expression within the wing imaginal disc cells of these larvae.

To determine whether Pacman expression was required throughout the wing imaginal disc, the same experiment was carried out but using the nub-GAL4 driver, which drives specifically within the wing pouch region of the disc (Figure 3.8D and E). As can be seen from Figure 3.9D, wing imaginal disc size in $y^1 pcm^{14}/Y$; $UAS-pcm^{WT}/nub$-GAL4 L3 larvae was partially rescued in size, in that it was significantly larger than $pcm^{14}/Y$ larvae, but also significantly smaller than $pcm^{WT}$ and $y^1 pcm^{14}/Y$; $UAS-pcm^{WT}/+$; 69B-GAL4/+ larvae. Therefore expression of pacman just in the wing pouch region of the disc in $pcm^{14}$ larvae was sufficient to rescue the size of the discs from 45% to 75% the size of wild-type. As a 45% to 100% increase in wing imaginal disc size would be regarded as 100% rescue, a 45% to 75% increase should be described as a 55% rescue ($(75-45)/(100-45)*100$). As the wing pouch region of the disc is roughly half the size of the disc, it therefore seems that pacman is required equally throughout the disc.
Figure 3.9. Expression of *pacman* throughout the wing imaginal discs of *pcm14* larvae rescues the reduced size of wing imaginal discs.

Diagrammatic representation of the cross set up to generate *y1 pcm14/Y; UAS-pcmWT/++; 69B-GAL4/+* L3 larvae in which *pacman* would be expressed throughout the wing imaginal discs of *pcm14* larvae. As non-disjunction is occurring in the *y1 pcm14/Y; UAS-pcmWT/CyO-GFP* stock the virgin females used for this cross will be either XX or XXY. All the possible genotypes from this cross are shown. *y1 pcm14/Y; UAS-pcmWT/++; 69B-GAL4/+* L3 larvae can be selected by selecting against the GFP present in the FM7i-GFP balancer and for the brown mouth part phenotype caused by the recessive *y1* marker. (B and C) Representative wild-type and *pcm14* wing imaginal discs. Scale bar represents 100µm. (D) The mean size of *pcm14* wing imaginal discs is 45% the size of wild-type. This phenotype can be rescued by expressing a *UAS-pcmWT* construct throughout the wing imaginal disc cells using the 69B-GAL4 driver. Driving *UAS-pcmWT* expression with *nub-GAL4* partially rescues this phenotype to 75% the size of wild-type. Expressing a *UAS-pcmND* construct throughout the disc reduces the mean wing disc size to 20% of wild-type (*n*≥31). *p*<0.001 for all comparisons unless indicated, ns = not significant, error bars represent 95% confidence limits.
What is interesting about this result is the observation that the overall size of the y¹ pcm¹⁴/Y; UAS-pcmWT/nub-GAL4 wing imaginal discs was still proportionally correct, in that the wing pouch region of the disc was 75% the size of wild-type and the thorax region of the disc was also 75% the size of wild-type, despite wild-type pacman expression being restricted to the wing pouch region of the disc. This suggests that the disc is developing as a whole unit and that the cells are communicating with each other to achieve this, presumably through the well characterised morphogen gradients of Dpp and Wingless.

3.8 The exoribonuclease activity of Pacman is essential for correct wing imaginal disc development

As mentioned above, Pacman also has the ability to act as a decapping factor. Therefore, it is important to determine whether the requirement of wing imaginal disc cells for Pacman is due to the exoribonuclease activity of Pacman or its role as a decapping factor. In order to determine whether the exoribonuclease function of Pacman is essential for wing imaginal disc development, wing imaginal disc size was measured in y¹ pcm¹⁴/Y; UAS-pcm¹⁰/++; 69B-GAL4/+ L3 larvae. As can be seen from Figure 3.9D, these discs were actually significantly smaller than pcm¹⁴ wing imaginal discs, being 20% the size of wild-type. Therefore expressing a nuclease dead Pacman in wing imaginal disc cells of pcm¹⁴ larvae has a dominant negative effect on its wing imaginal disc phenotype. This will be discussed in the discussion for this chapter.
3.9 Knockdown of *pacman* expression in wing imaginal disc cells using RNAi does not reduce the size of the wing imaginal discs

To further investigate the requirement of wing imaginal disc cells for Pacman, the GAL4/UAS system was used to knock down *pacman* specifically within the wing imaginal disc, using RNAi. As can be seen in Figure 3.4, Pacman protein is normally expressed ubiquitously throughout the disc. Use of the 69B-GAL4 driver, to drive expression of the UAS-pcmRNAi construct throughout the disc did not significantly reduce the size of the wing imaginal discs (Figure 3.10). This was not expected as the wing imaginal discs are significantly smaller in the pcm5 (82% the size of wild-type) and pcm14 (45% the size of wild-type) wing imaginal discs. The extent of Pacman knockdown in the 69B-GAL4/UAS-pcmRNAi wing imaginal discs was quantified using Western blotting and shown to be knocked down almost 16 fold compared to the UAS-pcmRNAi control discs (Figure 3.11).

*pacman* was also knocked down specifically in the wing pouch of the disc using the nub-GAL4 driver. nub-GAL4/+; UAS-pcmRNAi/+ wing imaginal discs were also not significantly smaller than the parental controls (Figure 3.10).

This result was unexpected and cannot be fully explained with the results presented in this thesis. However, one possible reason for this result could be to do with the decapping activity of Pacman, which is likely completely abolished in the *pcm*5 and *pcm*14 mutants which lack the Dcp1 binding motif, but may still be present at low levels when *pacman* is knocked down using RNAi. This will be discussed further in the discussion of this chapter.
Figure 3.10. Wing imaginal disc size is not reduced when pacman is knocked down using RNAi.

Wing imaginal disc size was not significantly reduced in 69B-GAL4/UAS-pcmRNAi or nub-GAL4/+; UAS-pcmRNAi/+ L3 larvae when compared with the UAS-pcmRNAi or nub-GAL4/+; 69B-GAL4/+ parental controls. n≥27. Error bars represent 95% confidence limits. p>0.05 for all comparisons.

Figure 3.11. Pacman is effectively knocked down in 69B-GAL4/UAS-pcmRNAi wing imaginal discs.

(A) Western blot probed with anti-Pacman for 69B-GAL4/UAS-pcmRNAi and UAS-pcmRNAi wing imaginal disc samples. Tubulin is used as a loading control. (B) Pacman expression is knocked down almost 16 fold in 69B-GAL4/UAS-pcmRNAi wing imaginal discs, when compared to the UAS-pcmRNAi parental control (n=3, error bars represent standard error).
3.10 Knockdown of *pacman* expression in specific domains of the wing disc results in loss of tissue in the corresponding domains of the adult wing

To further investigate the requirement of wing imaginal disc cells for Pacman, wing size of adults in which Pacman has been knocked down within specific compartments of the disc was measured. Wing size in 69B-GAL4/UAS-pcmRNAi adults was 69% the size of UAS-pcmRNAi control wings (Figure 3.12). These wings also displayed wing vein abnormalities, including loss of the anterior cross vein (94%), shortened L5 (8%) and shortened posterior cross vein (6%); (n=49). However, the shortened L5 was also observed in the nub-GAL4/+; 69B-GAL4/+ parental control (11%), so is unlikely to be an effect of *pacman* knockdown.

*pacman* expression was also knocked down specifically in the wing pouch of the disc using the nub-GAL4 driver. This knockdown also resulted in wings significantly smaller than the parental controls (72% the size of UAS-pcmRNAi control wings) (Figure 3.12), and also displayed wing vein abnormalities such as loss of the anterior cross vein (100%), and a shortened L5 vein (84%); (n=33).

In addition, *pacman* expression was knocked down specifically in the posterior compartment of the discs using the en-GAL4 driver (Figure 3.8F-G). The anterior compartment should have wild-type levels of Pacman and should therefore act as an internal control. Figure 3.12D and F show that the wings from these flies have distinct defects in the posterior of the adult wing (99% penetrance). The most common phenotypes recorded were blisters (66%), notches/loss of tissue (62%), and wing veins abnormalities, such as shortened L5 (19%) or branching of the posterior cross vein (13%) (n=818). The anterior of the wing was unaffected and developed normally (Figure 3.12D). These phenotypes were not observed in the engrailed-GAL4 or UAS-pcmRNAi parental strains.

These RNAi experiments provide further support to the hypothesis that Pacman is required autonomously for wing imaginal disc growth and development.
3.11 Leg, eye and haltere imaginal discs are significantly smaller in \(pcm^{14}\) L3 larvae compared to wild-type

In order to determine whether this growth phenotype was specific to the wing imaginal discs or whether all imaginal discs were affected, the size of the metathoracic leg, haltere and eye imaginal discs was measured in wild-type and \(pcm^{14}\) L3 larvae. The metathoracic leg imaginal discs were 59% the size of wild-type (n=21) (Figure 3.13A); the haltere discs were 47% the size of wild-type (n=21) (Figure 3.13B) and the eye discs were 60% the size of wild-type (n=16) (Figure 3.13C), demonstrating that this phenotype is not specific to the wing imaginal discs. Therefore Pacman appears to be involved in the growth and differentiation of all of these imaginal discs. During my PhD, however, I chose to concentrate my investigations on the role of Pacman in the wing imaginal disc, as the development and differentiation of this disc is better characterised than that of other discs.
Figure 3.13. *pcm*14 L3 larvae have significantly smaller metathoracic leg, haltere and eye imaginal discs.

The mean size of *pcm*14 metathoracic leg discs (A) is 59% the size of wild-type (n≥21); *pcm*14 haltere discs (B) are 47% the size of wild-type (n≥21) and *pcm*14 eye discs (C) are 60% the size of wild-type (n≥16). p<0.001 for all comparisons, error bars represent 95% confidence limits.
3.12 pcm\textsuperscript{14} larvae are not significantly smaller than wild-type

In pcm\textsuperscript{14} mutants, the imaginal discs could be smaller because the pcm\textsuperscript{14} mutation specifically affects the growth of the imaginal discs or because it effects the overall size of the larva. To differentiate between these possibilities, the growth rate of wild-type and pcm\textsuperscript{14} male larvae was compared. To achieve this, the surface area of the larvae was measured at 24h intervals and the results were plotted onto a graph (Figure 3.14A). The growth rates of these larvae are identical, suggesting that Pacman specifically affects the growth of the imaginal discs rather than the growth of the whole larva.

3.13 pcm\textsuperscript{14} larvae are delayed in development

During these experiments, it was observed that pcm\textsuperscript{14} larval development is significantly delayed. In order to quantify this, the larval time (time between the larva hatching from the embryo until the start of pupariation) was measured for pcm\textsuperscript{14} and pcm\textsuperscript{WT}. Figure 3.14B clearly illustrates the delay in pcm\textsuperscript{14} larval development, in that the majority of pcm\textsuperscript{14} larvae begin pupariation on average 36h later than pcm\textsuperscript{WT} larvae. Pupariation occurred at 136 hours after egg lay (AEL) (112 hours larval time plus 24 hours embryogenesis) in pcm\textsuperscript{WT} compared to 168 hours AEL (144 hours larval time plus 24 hours embryogenesis) in pcm\textsuperscript{14} mutants.

In order to determine whether pcm\textsuperscript{14} larvae continue to feed and grow during this extended larval time, the weight of pcm\textsuperscript{WT} and pcm\textsuperscript{14} larvae just prior to pupariation was measured. As can be seen from Figure 3.14C, pcm\textsuperscript{14} larvae were significantly larger than pcm\textsuperscript{WT}, despite the size and growth rates being the same throughout earlier larval development, demonstrating that pcm\textsuperscript{14} larvae do indeed continue to grow and feed during their extended larval time. During this time, the pcm\textsuperscript{14} wing imaginal discs also grew substantially in size (Figure 3.14D), from 45% to 66% the size of wild-type. This suggests that the reduced size of the wing imaginal discs is causing pcm\textsuperscript{14} larvae to reach critical size later in development (Stieper et al, 2008).
Figure 3.14. *pcm*\textsuperscript{14} larvae are not significantly smaller than *pcm*\textsuperscript{WT} larvae, but are delayed in development.  

(A) The growth rate of *pcm*\textsuperscript{14} larvae is not significantly different to wild-type (n≥16). (B) Onset of pupariation in *pcm*\textsuperscript{14} larvae is delayed by around 32 hours compared to wild-type (n≥42). (C) The mean weight of *pcm*\textsuperscript{14} larvae just prior to pupariation (152 hours) is 120% the mean weight of wild-type larvae just prior to pupariation (120 hours). (n≥35, p<0.0001). (D) During the extra 32 hours of development that *pcm*\textsuperscript{14} larvae undergo, the size of the wing imaginal discs increases from 45% to 66% the size of wild-type. (n≥30, p<0.001 for all comparisons). Error bars represent 95% confidence limits.
3.14 pcm**14** wing imaginal disc development is delayed

To further investigate the wing imaginal disc phenotype of pcm**14** mutants, the developmental patterning of the discs was measured. A common technique to determine the physiological age of wing imaginal discs is to stain the discs with an antibody for a well known morphogen involved in the patterning of the discs. Wingless is a well characterised morphogen involved in patterning during embryogenesis and during wing imaginal disc development (Couso et al, 1993). In mature wild-type L3 wing imaginal discs at 120h into development, the expression pattern of Wingless consists of a stripe of expression across the DV boundary, two concentric rings of expression surrounding the wing pouch of the disc, and a patch of expression at the tip of the thorax region of the disc (Couso et al, 1994).

pcm**WT** wing imaginal discs at 120h were stained with Wingless and the expected pattern of expression was indeed observed (Figure 3.15A). However, pcm**14** discs stained for Wingless at 120 hours, did not display this pattern of expression (Figure 3.15B). Instead, the pattern of these discs consisted of one ring of expression surrounding the wing pouch and there was diffuse Wingless expression within the wing pouch region itself, showing that these wing discs are immature. However, at 152 hours, when the majority of the mutant larvae are about to pupate, the pattern of Wingless expression was similar to wild-type (Figure 3.15C).

Therefore the delay in development of the wing imaginal discs correlates with the delay in larval development. One could therefore hypothesise that the delay in pupariation in pcm**14** mutants is to allow the growth and patterning of the wing discs (and most likely the other affected discs) to “catch up” with the growth and patterning that would normally be expected at this point in wild-type development in order to reach a “critical state” (Stieper et al, 2008).
Figure 3.15. Wing imaginal disc development in pcm14 larvae is morphologically delayed by 32 hours as determined by Wingless staining. (A) Wild-type wing imaginal disc at 120 hours displaying the correct pattern of Wingless. (B) pcm14 wing imaginal disc at 120 hours does not display the correct pattern of expression for this time point. The expression is more diffuse throughout the wing pouch and does not contain the two rings of expression surrounding the wing pouch. (C) pcm14 wing imaginal disc at 152 hours displaying the pattern of Wingless expression seen in wild-type at 120 hours. Scale bars represent 100µm.
3.15 *pacman* expression is critical for wing imaginal disc development during early L3

It has been determined that Pacman is required throughout the wing imaginal disc for correct wing development to occur. To investigate this further, the temporal requirement of Pacman was explored. Early experiments investigating the developmental requirement of Pacman in *Drosophila* showed that both *pacman* mRNA (Till et al, 1998) and Pacman protein (Grima et al, 2008) are differentially expressed during development.

In order to investigate this, the GAL4/UAS system was again used, but the temperature sensitive repressor of GAL4, GAL80<sup>ts</sup>, was also introduced. GAL80<sup>ts</sup> is active at 19°C and will bind and repress the GAL4 protein. Therefore at 19°C, no GAL4 mediated transcription of the UAS-<i>pcm</i><sup>RNAi</sup> will occur and therefore *pacman* expression will not be knocked down. At 29°C, the GAL80<sup>ts</sup> protein becomes inactive and can no longer bind to GAL4, allowing GAL4 mediated transcription of the UAS-<i>pcm</i><sup>RNAi</sup> to occur causing *pacman* to be knocked down (Leung & Waddell, 2004).

In order to accurately carry out this experiment, the perdurance of the system must first be calculated. This is the time it takes for Pacman protein to be knocked down, once the developing larvae have been moved from 19°C to 29°C. First of all the GAL80<sup>ts</sup> protein has to become completely inactivated, after which GAL4 must activate transcription of the UAS-<i>pcm</i><sup>RNAi</sup>, which then targets *pacman* mRNA for decay. Endogenous Pacman protein must also then be degraded before the cell is effectively knocked down of Pacman. The perdurance of the protein must therefore be calculated before designing the experiment.

Levels of Pacman were measured in *en-GAL4/+; UAS-<i>pcm</i><sup>RNAi</sup>/GAL80<sup>ts</sup> larvae at 19°C and 29°C and at 24h, 32h and 40h after being moved from 19°C to 29°C. Controls were used to test that the GAL80<sup>ts</sup> is effectively inhibiting GAL4 at 19°C and that it is inactive at 29°C.

As can be seen from Figure 3.16, the GAL80<sup>ts</sup> is effectively repressing the GAL4 at 19°C as levels of Pacman are the same in these larvae with and without the *en-GAL4* driver. Also, the GAL80<sup>ts</sup> is effectively inactivated at 29°C as Pacman expression is the same in these larvae with and without the GAL80<sup>ts</sup>.

Pacman expression is not knocked down 100% in *en-GAL4/+; UAS-<i>pcm</i><sup>RNAi</sup>/GAL80<sup>ts</sup> larvae 24h after being moved to 29°C but is after 32h. Therefore, the perdurance can be calculated as between 24h and 32h. Experiments were therefore setting up allowing for at least 24h for Pacman to be knocked down after moving the larvae to 29°C.
Figure 3.16. Pacman is effectively knocked down in en-GAL4/+; UAS-pcmRNAi/GAL80ts larvae 24-32h after temperature increase from 19°C to 29°C. Levels of Pacman expression was measured in en-GAL4/+; UAS-pcmRNAi/GAL80ts larvae at 24h, 32h and 40h after temperature increase from 19°C to 29°C as well as controls at either 19°C or 29°C. Expression was not different between en-GAL4/+; UAS-pcmRNAi/GAL80ts, larvae at 19°C and the negative control, CyO-GFP/+; UAS-pcmRNAi/GAL80ts at 19°C, demonstrating that GAL80ts is effectively inhibiting GAL4 at 19°C. The expression is also not different between en-GAL4/+; UAS-pcmRNAi/GAL80ts and the positive control, en-GAL4/+; UAS-pcmRNAi/TM6 at 29°C, demonstrating that the GAL80ts is effectively inactivated at 29°C. Expression of Pacman is not completely knocked down by 24h but is by 32h, demonstrating that the perdurance is between 24h and 32h.

<table>
<thead>
<tr>
<th></th>
<th>19°C</th>
<th>19°C</th>
<th>19°C</th>
<th>19-29°C</th>
<th>19-29°C</th>
<th>19-29°C</th>
<th>19-29°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>en-GAL4</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UAS-pcmRNAi</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GAL80ts</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

---

Pacman  

Tubulin  

![Pacman and Tubulin Blots](image.png)
Taking this result into account, it was decided that Pacman would be knocked down using en-GAL4 at the start of L2, start of L3, mid L3 and the start of pupariation. As the perdurance is at least 24h, it would not be feasible to knock down Pacman at the start of L1 as embryogenesis takes 24h, and so the larvae would have to be moved to 29°C at time point 0h which would actually mean that Pacman would be knocked down from the start of embryogenesis, as GAL80ts would never be active in the first place.

The experiment was set up as in Figure 3.17. The crosses were set up at 19°C (except the knock down control which was at 29°C), with an 8h egg lay period so that enough eggs could be laid for analysis, but also so that the developing larvae were all of roughly the same age. Vials containing the offspring of the cross were then moved from 19°C to 29°C at 48h intervals (at 19°C each stage of development takes 48h as opposed to 24h at 25°C or 29°C) with a wild-type control staying at 19°C for the entire development. Wings of en-GAL4/++; UAS-pcm^RNAi/GAL80ts adult flies were then dissected and photographed and the size of the anterior and posterior compartments was measured.

As can be seen from Figure 3.18A, the size of the anterior compartment was not significantly different between any of the time periods, except the wild-type control, which had a significantly larger anterior compartment than all of the other time periods. The most likely explanation for this is the fact that these flies spent the entire time at 19°C, whereas the other time periods spent the majority of the time at 29°C. To overcome this, the mean size of the posterior compartment was normalised to the mean size of the anterior compartment for each time period (Figure 3.18B).

As expected, the normalised size of the posterior compartment was significantly smaller (51%) in the knocked down control compared to wild-type control. The size of the normalised posterior compartment was not significantly different between either the knock down control or flies in which Pacman was knocked down at the start of L2 or the start of L3. The normalised posterior compartment in flies where Pacman was knocked down mid L3 were significantly different to all other time periods, being intermediate to the knock down control and the wild-type control. The posterior compartment of flies where Pacman was knocked down at the start of pupation was not significantly different to the wild-type control.
Figure 3.17. Diagrammatic representation of the temperature shifts carried out during the GAL80ts experiment. 

(A) Diagrammatic representation of the cross carried out to generate en-GAL4/+; UAS-pcmRNAi/GAL80ts flies. (B) The wild-type control is placed at 19°C and the knockdown control at 29°C for the entire development. In order to knockdown Pacman at the start of L2, vials containing the larvae were moved from 19°C to 29°C 2 days after egg lay (AEL) so that by the time Pacman was effectively knocked down (24-32h perdurance effect), the larvae would be at the start of L2 (light red line). In order to knock down Pacman at the start of L3 the vials containing the larvae were moved from 19°C to 29°C 4 days AEL (light green line), mid L3 was 6 days AEL (blue line) and start of pupariation was 8 days AEL (dark red line).
A

![Bar chart showing the comparison of anterior wing size across different stages of development with standard errors.

Stage of development at which pcm is knocked down.

B

![Bar chart showing the comparison of posterior/anal wing size across different stages of development with standard errors.

Stage of development at which pcm is knocked down.

C

Images illustrating the effects of pcm knockdown at different stages of development:

- Knockdown control
- Mid L3
- Start of L2
- Start of pupation
- Start of L3
- Wild-type control
Figure 3.18. Wing size is reduced and wing venation phenotypes are observed only in flies in which Pacman has been knocked down before late L3.

(Figure on previous page)

(A) The mean size of the anterior wing compartment of en-GAL4/+; UAS-pcmRNAi/GAL80ts flies in which Pacman was knocked down at different stages of development. (n≥12. Error bars represent 95% confidence limits. P>0.05 for all comparisons except between the wild-type control and all other comparisons.) (B) The mean size of the posterior compartment normalised to the mean size of the anterior compartment. (n≥12. Error bars represent 95% confidence intervals. P<0.001 for all comparisons unless otherwise stated. ns=not significantly different.) (C) Wing venation phenotypes were observed in the wings of the knockdown control flies (as expected) and flies in which Pacman was knocked down from the start of L2 and the start of L3. These were restricted to the posterior compartment except for "Extra ACV". Wing venation phenotypes were not observed in the wild-type control (as expected) or flies where Pacman was knocked down from mid L3 or the start of pupariation. (Scale bars represent 200µM).

In addition, these wings were also scored for any wing venation phenotypes. Phenotypes were observed in the knockdown control and the flies in which Pacman was knocked down at the start of L2 and the start of L3 but not mid L3 or the start of pupariation or the wild-type control (Figure 3.18C). This correlates with whether the posterior compartment of the wing was significantly reduced in size.

The above results are summarised in the table in Figure 3.19. As no phenotype was observed when Pacman was knocked down from the start of pupariation, it can be concluded that Pacman expression is not required after larval development is complete for correct wing development to occur. This implies that Pacman is required for the growth of the wing imaginal discs rather than their differentiation, as the discs proliferate during larval development and differentiate during pupation (Neto-Silva et al, 2009). Furthermore, the severity of the phenotype is no more severe when Pacman is knocked down throughout development compared with when Pacman is knocked down from the start of L3, which implies that Pacman expression is not essential before the L3 period for correct wing development to occur. Also the phenotype is less severe when Pacman is knocked down from mid L3 when compared with the start of L3 as the posterior compartment is larger and there are no wing venation phenotypes. Therefore, it appears that the critical stage of development in which Pacman expression is essential for the correct development of the wings is during early L3.
Figure 3.19. Pacman expression is critical during early L3 for correct wing development to occur.

The results from the en-GAL4/+; UAS-pcmRNAi/GAL80ts experiment are summarised by the table, which shows at what stages Pacman is either knocked down or active and whether any phenotypes are observed.
3.16 Pacman expression is not required during embryogenesis or L1 for the viability of the organism

In order to determine the temporal requirement of Pacman for the viability of the organism it was decided that a similar experiment would be carried out as above, but investigating the act-GAL4/+; UAS-pcmRNAI/+ lethality. act-GAL4 drives expression throughout the larva and act-GAL4 driven expression of the UAS-pcmRNAI construct has been shown previously to result in lethality (Figure 3.6C). act-GAL4/+; UAS-pcmRNAI/GAL80ts flies which have developed at 19°C eclosed as adults (Figure 3.20B), demonstrating that at 19°C the GAL80ts is effectively inhibiting the GAL4 protein and pacman expression is at wild-type levels. act-GAL4/+; UAS-pcmRNAI/GAL80ts flies which have developed at 29°C however do not survive (Figure 3.20B), which demonstrates that at 29°C the GAL80ts is inactivated and Pacman is effectively knocked down throughout the larvae. act-GAL4/+; UAS-pcmRNAI/GAL80ts flies which are moved from 19°C to 29°C 48h after egg lay (AEL) are also pupal lethal. This shows that despite Pacman expression being wild-type during embryogenesis and L1, and effectively knocked down from the start of L2, the lethality is not rescued. However act-GAL4/+; UAS-pcmRNAI/GAL80ts flies which are moved from 29°C to 19°C 24h after development, do indeed eclose as adults. Therefore, when Pacman is knocked down during embryogenesis and L1 but at wild-type levels from the start of L2, these flies survive to adulthood. This experiment shows that Pacman expression is not required during the embryonic or L1 stages of development (Figure 3.20).

Analysis of this result however does need to consider the fact that pacman is maternally contributed (Till et al, 1998). Also there is no transcription during the first 8h of embryonic development (Edgar & Schubiger, 1986), which means there will be no transcription of the UAS-pcmRNAI and so pacman expression will not be knocked down during this time. Therefore, it cannot be ruled out that Pacman is required during this time, and the fact it is maternally contributed suggests that it probably is. In order to investigate this, a maternal null of pacman would need to be generated to completely remove any pacman expression during embryogenesis.
Figure 3.20. Pacman expression is not required during embryogenesis or L1 for the viability of the organism.

(A) Diagrammatic representation of the cross carried out to generate ac-GAL4/+; UAS-pcmRNAi/GAL80ts flies. (B) The results from the ac-GAL4/+; UAS-pcmRNAi/GAL80ts experiment investigating lethality are summarised by the table, which shows at what stages Pacman is either knocked down or active and whether any lethality is rescued.
3.17 Chapter summary

The aims of this chapter have been successfully addressed in order to further characterise the \(pcm^{14}\) mutant phenotypes. The evidence presented here shows that the pupal lethality of \(pcm^{14}\) is a direct consequence of the lack of functional Pacman expression in this mutant and the exoribonuclease function of Pacman is essential for the organisms viability. The reduced size of the wing imaginal discs in \(pcm^{14}\) L3 larvae is caused by lack of Pacman within the wing imaginal disc cells, demonstrating a requirement of Pacman for wing imaginal disc cells to grow and develop correctly. This phenotype is not however restricted to the growth of the wing imaginal discs as all imaginal discs which were measured in size (metathoracic leg, haltere and eye) were also significantly smaller than wild-type. This growth defect was not simply because the whole larva was growing slower, as the growth rates were not significantly different in \(pcm^{14}\) and \(pcm^{WT}\) larvae. Despite this, the \(pcm^{14}\) larvae were developmentally delayed, in that it took on average an extra 32 hours of development to pupate when compared with wild-type. During this extra period of development, the larvae continued to feed and grow, as \(pcm^{14}\) larvae just prior to pupariation, weighed significantly more (120%) than wild-type. During this period the size of the wing imaginal discs also grew significantly, from 45% to 66% the size of wild-type. \(pcm^{14}\) larvae also required this extra 32 hours of development for the correct patterning of Wingless to be observed in mature wild-type L3 larvae, demonstrating that the wing imaginal disc development in \(pcm^{14}\) larvae is developmentally delayed, in addition to their growth being retarded. It can also be concluded from these results that the critical period of development in which Pacman activity is required for correct wing development to occur is early L3.

3.18 Acknowledgements

Experiments scoring wing phenotypes in \(en\text{-GAL4/+;UAS-pcm^{RNAi}}/\) adults were carried out by Hannah Parker. Measuring and scoring wings in \(nub\text{-GAL4/+;UAS-pcm^{RNAi}}/\) and \(69B\text{-GAL4/UAS-pcm^{RNAi}}/+\) adults was carried out by Karen Scruby and Clare Rizzo-Singh. Larval size, weight and timings were measured by Sam Crossman. GAL80\textsuperscript{ts} experiments were carried out by Ben Towler. These were all carried out under my supervision.
3.19 Chapter discussion

3.19.1 Why is $pcm^{14}$ a null allele?

Although the genetic and molecular data presented in this chapter show that $pcm^{14}$ is a null allele, the exact explanation for this is unclear. The level of transcription from the pacman locus is unaffected in $pcm^{14}$ cells as the levels of $pcm^{14}$ mRNA were not significantly different to wild-type (Figure 3.1C). As the antibody for Pacman binds the region deleted in $pcm^{14}$ (Figure 3.1A), it is not possible to determine whether the $pcm^{14}$ mRNA is translated by Western blotting.

Recently in the lab, Dr. Chris Jones has performed mRNA-Sequencing (RNA-Seq) using RNA extracted from $pcm^{WT}$ and $pcm^{14}$ wing imaginal discs. The reads mapping to the pacman locus are shown in Figure 3.21. This confirms that mRNA is being transcribed from the $pcm^{14}$ allele up until the start of the deletion. It also shows that transcription continues past the deletion, and could potentially be transcribing into the convergently transcribed gene Nat1. This observation is based on the fact that reads are mapped to the intergenic region between pacman and Nat1, which is not seen in wild-type. However, these reads appear to map to the 3′ region of the non-coding RNA CR43260 which is not deleted by the $pcm^{14}$ deletion, with very few reads representing the genomic region between CR43260 and Nat1. As this RNA is poly(A) selected and as CR43260 is located in the same orientation as pacman, this suggests that transcription occurs through the poly(A) cleavage site of CR43260, after which cleavage occurs, allowing transcription termination and polyadenylation to occur. This would suggest that Nat1 would be unaffected, but as these results only represent polyadenylated RNA, it does not rule out the possibility that transcription may also continue through CR43260 and into Nat1, for transcripts that do not undergo polyadenylation.

This experiment supports the evidence that the $pcm^{14}$ mRNA is transcribed at wild-type levels, although this cannot be accurately quantified, as the algorithms used for quantification assumes that the whole gene region is present for both samples. However, as roughly half the pacman gene is deleted in $pcm^{14}$, one would expect half as many reads to map to this gene. This is indeed what is observed, suggesting no down-regulation of transcription.
Figure 3.21. Annotated screen shot of RNA-Seq results showing mapped regions of transcripts at the *pacman* locus for *pcm* WT and *pcm* 14 poly(A) selected wing imaginal disc RNA.

As expected, no transcripts map to the region known to be deleted in *pcm* 14. *pacman* is represented in the reverse orientation with the 5’ end to the right. Transcripts mapping to the genomic region downstream of the region deleted in *pcm* 14 most likely represent the 3’ end of *CR43260* as there is a substantial dip in the number of reads between this peak and before the start site of *Nat1*. This work was carried out by Dr. Chris Jones in the Newbury lab.
As the pcm\textsuperscript{14} mRNA transcript is therefore not down-regulated and is likely to be capped and polyadenylated, it is also likely to be translated. However, despite the catalytic domain still being present in this allele (Figure 3.1A), it has been shown that any protein produced from this allele is not able to degrade the cleaved 3\textquotesingle fragment of the Adh\textsuperscript{I6} allele (Figure 3.3B). Although this does not show for certain that pcm\textsuperscript{14} protein cannot degrade other RNAs, this does support the argument that there is no detectable cytoplasmic 5\textquotesingle-3\textquotesingle catalytic exoribonuclease activity in pcm\textsuperscript{14} cells, as Pacman/Xrn1 is not thought to show any specificity between substrates with a 5\textquotesingle phosphate group (Nagarajan et al, 2013). This supports the findings from (Page et al, 1998), who showed that large C terminal deletions reduced the exoribonuclease activity of Xrn1 in S. cerevisiae to less than 10%, despite not effecting the catalytic domain.

The two most likely explanations for Pacman protein lacking exoribonuclease activity in pcm\textsuperscript{14} cells is that either the protein is unstable and is being rapidly degraded, or that the N terminal catalytic domain relies on the C terminal domain being present in order to function correctly. This could either be a structural property, or that the C terminal domain binds co-factors, such as Dcp1, to "feed" the catalytic domain its substrates.

Another possible explanation that cannot be excluded is that the 3\textquotesingle end of CR43260, which is being transcribed as part of the pcm\textsuperscript{14} mRNA transcript, is inhibiting the catalytic activity of the Pacman protein encoded in pcm\textsuperscript{14} cells. However, this does not seem to be acting in a dominant negative manner as expression of the UAS-pcm\textsuperscript{WT} construct throughout the wing imaginal discs completely rescues the size of the wing imaginal discs in pcm\textsuperscript{14} L3 larvae.

### 3.19.2 Are the neighbouring genes CR43260 and Nat1 affected by the pcm\textsuperscript{14} mutation?

The failure of transcription termination can have deleterious effects on neighbouring genes in that it has been shown that for convergent genes, the failure of termination in one gene can cause transcriptional collision or dsRNA formation, which would ultimately reduce expression of the neighbouring gene (Gullerova & Proudfoot, 2008). It is therefore essential to be certain that the phenotypes observed in the pcm\textsuperscript{14} mutants are a direct consequence on the lack of pacman expression and are not contributed by an interference in the expression of either Nat1 or also the deletion in CR43260.
**Nat1** is predicted to possess acetyl transferase activity and has been implicated in neurogenesis (FlyBase, 1992). It is expressed at moderate to high levels in all larval and adult tissues/organs except the salivary glands (Graveley et al, 2011a; Graveley et al, 2011b) and has been shown to be essential for viability (Peter et al, 2002). The expression of **Nat1** in **pcm** was shown not to be significantly different to wild-type from the RNA-Seq data, which strongly supports the argument that **Nat1** is unaffected by the **pcm** mutation.

The function of **CR43260** is unknown and was only recently annotated as a gene. The ModENCODE data on FlyBase shows that this gene is moderately expressed in the testis and fat body and lowly expressed in the imaginal discs of L3 larvae (Graveley et al, 2011a; Graveley et al, 2011b). However, the RNA-Seq data did not detect **CR43260** in wild-type L3 wing imaginal discs, suggesting that it is not required for their development.

The strongest piece of evidence to support the argument that the deletion of **CR43260** and any possible interference with **Nat1** is not contributing to the **pcm** phenotypes is that both the pupal lethality and reduced wing imaginal disc size of **pcm** mutants can be rescued 100% by the expression of wild-type **pacman** cDNA. Furthermore, when **pacman** is knocked down using RNAi in the wing imaginal disc cells the wings in the adult are significantly smaller and display wing venation defects. Also, the phenotypes observed in **pcm** mutants, in which 529bp of the 3' end of **Nat1** and the entire **CR43260** are deleted are less severe than phenotypes observed in **pcm** mutants, in which **Nat1** and **CR43260** are complete.

Further, more recent evidence to support this has been derived from the generation of a new null **pacman** allele named **pcm**. This allele was generated by imprecise excision of the P element **P[EP]pcm[G1726]**, located in the 5'UTR of **pacman**. **Nat1** and **CR43260** are unaffected in this allele and the observed phenotypes are the same as in **pcm** (personal communication with Chris Jones).

It can therefore be concluded that any effect that the **pcm** mutation is having on the expression of **Nat1** and **CR43260** is not contributing to the observed phenotypes in the **pcm** mutant larvae.

### 3.19.3 Why are wing imaginal discs not smaller when **pacman** is knocked down using RNAi?

The answer to the above question is not absolutely clear. It has been shown that levels of Pacman in 69B-GAL4/UAS-**pcm**RNAi wing imaginal disc cells is almost 16 fold less than in the
parental control UAS-pcm\textsuperscript{RNAi} wing imaginal disc cells, yet these wing imaginal discs are not significantly different in size. It could be argued that complete knockdown of Pacman is required to have an effect, but this is not the case as exoribonuclease activity in pcm\textsuperscript{5} larvae was shown to be roughly 66.7% functional, yet these wing imaginal discs are 82% the size of wild-type.

One possible explanation is that in pcm\textsuperscript{5} mutants decapping is likely to be impeded, as the Dcp1 binding motif is deleted in this mutation, in addition to exoribonuclease activity being reduced; whereas in pcm\textsuperscript{RNAi} discs, decapping may not be as strongly affected as low levels of Pacman will still be present within the cells.

Although 69B-GAL4/UAS-pcm\textsuperscript{RNAi} discs are the same size of wild-type, the wing size is significantly smaller than the parental controls, being 69% the size of the UAS-pcm\textsuperscript{RNAi} parental control. This therefore suggests that the critical period of Pacman expression is actually later in development than L3. This would however contradict the results obtained from the GAL80\textsuperscript{ts} experiment, which show that early L3 is the critical time period for Pacman expression in order for correct wing development to occur.

In order to investigate this further, the reason that pcm\textsuperscript{14} wing imaginal discs are reduced in size needs to be investigated. The most likely explanations for reduced growth is that there is an increase in apoptosis and/or a reduction in cell division. Also the cells could be smaller in size. If the reason is apoptosis, then it is possible that compensatory proliferation is also occurring (Fan & Bergmann, 2008a; Martin et al, 2009). If this is the case, there may be a difference in the ability of pcm\textsuperscript{14} and pcm\textsuperscript{RNAi} cells in inducing compensatory proliferation. The reason that the discs are not significantly different in size but the wings are is perhaps related to the L3 stage of development being the critical stage, in that this could be the final time period where the dead cells are able to be replaced by compensatory proliferation. This is supported by the fact that the discs are undergoing rapid growth during the larval stages in order to reach a final size and then differentiate during the pupal stage of development (Neto-Silva et al, 2009). The following chapter will investigate this further.

3.19.4 Expressing a "nuclease dead" Pacman throughout the wing imaginal discs in pcm\textsuperscript{14} larvae has a dominant negative effect on the wing imaginal disc phenotype.
Figure 3.9D shows that the size of wing imaginal discs in y¹ pcm¹⁴/Y; UAS-pcm⁰⁰/+; 69B-GAL4/+ L3 larvae (20% the size of wild-type) is significantly smaller than in pcm¹⁴/Y L3 larvae (45% the size of wild-type). Therefore, expression of this "nuclease dead" Pacman in pcm¹⁴ wing imaginal discs appears to be having a dominant negative effect. This could be a result of sequestration of the Decapping protein Dcp1 (which binds to a motif within the C-terminal unstructured domain, which would be present in the "nuclease dead" Pacman being expressed), together with lack of exonuclease activity. In Drosophila tissue culture cells, both knockdown and overexpression of Xrn1 inhibited both decapping and degradation of a reporter RNA leading to an accumulation of capped fragments (Braun et al, 2012). This was shown to be dependent on the Dcp1 binding domain within Xrn1 as overexpression of a "nuclease dead" Xrn1 had the same effect, whereas overexpression of Xrn1 which was had its Dcp1 binding domain deleted failed to show the same effect.

Therefore in pcm¹⁴ there is no cytoplasmic 5'-3' exoribonuclease activity and decapping should also be impaired as the Dcp1 binding domain is deleted in pcm¹⁴. However, overexpression of Pacman was shown to have stronger inhibition on decapping than knockdown of Pacman (Braun et al, 2012), so by overexpressing a "nuclease dead" Pacman in these cells would not rescue any exoribonuclease activity but could impair decapping further. This would lead to a greater increase in the accumulation of capped RNAs which are more likely to be translated and therefore more likely to show an effect at the phenotypic level.

This dominant negative effect of overexpressing Pacman is also supported by the results in Figure 3.6C that show that overexpression of both UAS-pcm¹⁴ and UAS-pcm⁰⁰ with act-GAL4 caused pupal lethality.

Another possible explanation for this result could be that the expression of the nuclease dead construct is inhibiting any decapping activity associated with the maternal contribution of Pacman. It is possible that Pacman protein translated from the maternal contribution of pacman mRNA will still be present in cells in very early wing imaginal discs in pcm¹⁴ L1 larvae. Therefore this could overlap with expression of the UAS-pcm⁰⁰ being driven by 69B-GAL4, which could inhibit decapping and the activity of Pacman in these cells. It would be difficult to test this without creating a maternal null mutant for pacman, but this could be the subject of future work. It is indeed likely that the phenotypes of a maternal null for pacman would be more severe than those observed in pcm¹⁴ mutants, although it could indeed be embryonic lethal.
4 Investigating the reduced growth phenotype of *pcm*\(^{14}\) wing imaginal discs

4.1 Introduction

In the previous chapter the main phenotypes of the null *pcm*\(^{14}\) mutant were characterised. The main phenotypes observed were the reduced growth of the wing, eye, leg and haltere imaginal discs and the delayed development of the wing imaginal discs, which correlated with the delayed development of the larva. This chapter investigates the reduced growth of the imaginal discs further, concentrating on the wing imaginal discs, as the development of the wing discs is better characterised than the other discs. The main question that will be addressed is whether the reduced size of the discs is a result of an increase in apoptosis or a reduction in cell division, or a combination of the two.

Apoptosis, also termed Programmed Cell Death (PCD), occurs in response to cell stress and during normal development of multi-cellular organisms (Meier et al, 2000a). The regulation of apoptosis is tightly controlled and many diseases are associated with loss of this control, such as cancer (Danial & Korsmeyer, 2004). Apoptosis occurs during development to ensure the removal of cells which are no longer required and occurs in a developmentally programmed manner (Meier et al, 2000a).

Control of apoptosis is coordinated with that of proliferation, with many signalling pathways implicated in the normal control of tissue growth involved in both processes (Danial and Korsmeyer 2004). *Drosophila* provides an excellent model system for the study of apoptosis because of its genetic traceability and the similarities of its apoptosis pathways to that of other organisms (Hay et al, 2004; Meier et al, 2000a; Salvesen & Abrams, 2004; Steller, 2008; Xu et al, 2009). In order to determine whether apoptosis is occurring in *pcm*\(^{14}\) wing imaginal discs, the discs will be stained with an anti-activated Caspase 3 antibody which labels cell undergoing apoptosis (Fan & Bergmann, 2010).

As mentioned, proliferation and apoptosis are tightly regulated and it is likely that if apoptosis is induced as a result of the *pcm*\(^{14}\) mutation, then compensatory proliferation may also be occurring to counteract this (Fan & Bergmann, 2008a; Martin et al, 2009). However, just as likely an explanation for the reduced growth of the wing imaginal discs in *pcm*\(^{14}\) mutants could be that cell division is down regulated. During the larval period of
development the discs grow from roughly 50 to 50,000 cells in order to reach a critical size so that the adult structures of the fly (wings and part of the thorax) can be formed (Neto-Silva et al, 2009). If cell division is impeded in pcm14 larvae then this would therefore cause the discs to be reduced in size and also to be delayed in development. In order to determine whether the rate of cell division is affected by the pcm14 mutation, the wing imaginal discs will be stained with an anti-phosphohistone H3 antibody which labels cells in M phase. From this a mitotic index can be calculated, by dividing the number of cells in M phase by the area of the disc.

In order to further characterise the reduced growth phenotype of pcm14 mutants, mosaic analysis will be used to investigate whether Pacman is required by wing imaginal disc cells in a cell autonomous manner. This is important as it has been shown that the imaginal discs coordinate their growth so that if growth perturbation is seen in one of the imaginal discs, then the other imaginal discs can slow down their own growth to compensate (Parker & Shingleton, 2011). It is therefore possible that wing imaginal disc growth is delayed in response to the reduced growth of the leg, eye or haltere imaginal discs being delayed in growth and development (Figure 3.13).

Mosaic analysis is a common genetic technique used in Drosophila to investigate whether a gene is involved in the regulation of growth (Xu & Rubin, 1993). This technique directly compares the growth rates of two genetically distinct cells in developing tissues. By inducing mitotic recombination in cells undergoing cell division, two daughter cells are produced which differ in their genetic material, both from each other and the parental cell. These cells can be distinguished from each other by the presence or absence of a fluorescent reporter such as GFP. These cells will then divide to form populations of cells termed clones. By examining the sizes of these clones, it is possible to determine if one genotype has a growth advantage over another (Figure 4.1).

This technique allows the functions of crucial genes, that when mutated would otherwise cause lethality during embryogenesis, to be investigated in later developmental stages. It is commonly used in Drosophila to determine whether certain genes regulate growth. In this chapter it is used to investigate the cell autonomous requirement of Pacman for the growth of wing imaginal disc cells.
Figure 4.1. Diagrammatic representation showing the principles of mosaic analysis.
(A) Site directed mitotic recombination is induced in all somatic cells undergoing DNA replication using the FLP/FRT system. When this occurs in cells heterozygous for the pcm14 mutation, at position 19A, two daughter cells are produced which are genetically distinct, both from each other and the parental cell. One of these daughter cells, termed the mutant daughter cell, is homozygous for the pcm14 mutation and the other, termed the wild-type daughter cell, is homozygous for the wild-type allele of pcm. All three of these cells can be distinguished from each other by the copy number of the mRFP reporter. The parental cells have one copy of the mRFP gene, the wild-type cells inherit two copies of the mRFP gene and the mutant cells have no mRFP gene. (B) These daughter cells will divide to form populations of cells termed clones. If the wild-type cells have a growth advantage over the mutant cells then the wild-type clones will be larger than the mutant clones.
4.2 Aims

In order to address the above questions the following techniques will be used.

- Mosaic analysis will be carried out to directly compare growth rates between wild-type and pcm14 cells.
- Immunocytochemistry will be used to determine whether the reduced growth of pcm14 wing imaginal discs is caused by an increase in apoptosis or a reduction in cell division (or a combination of the two).

4.3 Creating the w^{1118} pcm^{14} P{neoFRT}19A/FM7i stock

To investigate the requirement of Pacman for the growth and development of the wing imaginal disc cells, mosaic analysis was used. As previously described this technique involves comparing the growth rates of clones of cells with a mutant or wild-type homozygous genotype in a background of heterozygous cells.

In order to create clones of wild-type and mutant cells, site directed recombination is required to take place. For this, the FLP/FRT system was used (Theodosiou & Xu, 1998; Xu & Rubin, 1993). This system requires the Flippase Recognition Target (FRT) sequence to be inserted at the same position on homologous chromosomes, so that the Flippase (FLP) (under heat-shock control) can induce recombination at the FRT site. Therefore, the FRT sequence first had to be recombined onto the w^{1118} pcm^{14} chromosome, proximal to the pacman locus. The only available FRT site proximal to the pacman locus, which maps to cytological position 18C on the X chromosome, was located at cytological position 19A.

In order to create a chromosome containing both the pcm^{14} mutation and the FRT sequence at position 19A, recombination was required to take place between these two loci during meiosis (Figure 4.2A). To this end, y^{1} w^{1118} P[neoFRT]19A males were crossed to w^{1118} pcm^{14}/FM7i virgin females to generate w^{1118} pcm^{14}/y^{1} w^{1118} P[neoFRT]19A female offspring, in which this recombination event could occur. Following this, crosses were used to screen for offspring which contain the w^{1118} pcm^{14} P[neoFRT]19A chromosome. The crosses which were carried out to generate this stock are shown in Figure 4.2B and described below.
Figure 4.2. Creation of the $w^{1118}$ pcm14 $P$[neoFRT19A]/FM7i stock. 

(A) Diagrammatic representation of the recombination event required to take place in order to generate the $w^{1118}$ pcm14 $P$[neoFRT19A] chromosome. (B) Diagrammatic representation of the crosses carried out to generate the $w^{1118}$ pcm14 $P$[neoFRT19A]/FM7i stock. Regions of the genome in brackets represent parts which cannot be phenotypically determined at that stage of the screen. Crosses F0 and F1 were carried out in bulk to generate enough females for the F2, which was carried out with individual virgin females. 511 crosses were set up, of which 272 produced progeny and of those 105 contained a lethal mutation on the X chromosome. Of these, 4 produced $w^{1118}$ pcm14 $P$[neoFRT19A]/FM7i females in the offspring of the G418 screen in the F3 and one of these was used to set up a stable stock in the F4.
In the F0, \( w^{1118} \) \( pcm^{14} \)/\( FM7i \) virgin females were crossed to \( y^{1} w^{1118} \) \( P\{neoFRT\}19A \) males, in order to generate \( w^{1118} \) \( pcm^{14} \)/\( y^{1} \) \( w^{1118} \) \( P\{neoFRT\}19A \) females. These females were selected from the offspring by selecting for round eyed females, as all the other females from the cross will contain the \( FM7i \) balancer and so would have heart shaped eyes.

In the F1, \( w^{1118} \) \( pcm^{14} \)/\( y^{1} \) \( w^{1118} \) \( P\{neoFRT\}19A \) virgin females were crossed to \( FM7i/Y \) males. The offspring of this cross will all be genetically unique, as recombination would have occurred during meiosis in the mothers. These offspring will therefore either contain just the \( pcm^{14} \) mutation, just the \( P\{neoFRT\}19A \) insertion, both the \( pcm^{14} \) mutation and the \( P\{neoFRT\}19A \) insertion or neither. However, it was not possible to distinguish between these genotypes in the offspring of this cross.

In order to determine which flies had the \( pcm^{14} \) mutation, crosses were set up with individual virgin females being crossed to \( FM7i/Y \) males. As the distance between the \textit{pacman} locus and the \( P\{neoFRT\}19A \) insertion is only 2cM, the percentage of flies containing both the \( pcm^{14} \) mutation and the \( P\{neoFRT\}19A \) insertion will be roughly 1% (the recombination event will happen on average 1 in 50 times and there is a 50% chance of the desired chromosome being inherited by the offspring). To maximise the chances of recovering the desired chromosome, 511 virgin females from the offspring of the F2 were crossed to \( FM7i \) males in individual vials to screen for the presence of the \( pcm^{14} \) mutation.

Of these 511 crosses only 272 produced any progeny. The genotypes of the offspring from these crosses are shown. If the mothers from the cross did have the \( pcm^{14} \) mutation, then the only males present in the offspring of this cross should have Bar eyes. This is because \( pcm^{14} \) hemizygous males are pupal lethal. From the 272 crosses which produced progeny, 167 contained round eyed males in the progeny, which means there was no lethal mutation on the X chromosome of the mother. These crosses were therefore disposed of.

As the only offspring from this cross which are viable and fertile are identical to the parents, each cross has essentially set up a stable stock.

The remaining 105 stocks which did contain a lethal mutation on the X chromosome were then screened for the presence of the \textit{neo} resistance gene present in the \( P\{neoFRT\}19A \) insertion. By adding G418 to the food, only flies which contain the \( P\{neoFRT\}19A \) insertion will survive. The remaining 105 crosses were turned over into two new vials, one of which underwent the G418 screen and the other was used to create a stable stock. Only flies with the \( P\{neoFRT\}19A \) insertion will survive the G418 screen, therefore the only surviving offspring from these crosses will be \( y^{1} w^{1118} \) \( pcm^{14} \) \( P\{neoFRT\}19A/FM7i \) females, if the
$P_{neoFRT}19A$ is present. If the $P_{neoFRT}19A$ is not present then no offspring will survive. From the 105 crosses, only 4 produced any offspring, and all of these were of the desired genotype, which showed that the screen had worked correctly. One of these was used to set up the stable stock $w^{1118}pcm^{14}P_{neoFRT}19A/FM7i$.

It would also have been possible for the $y^1$ marker also to be present in this stock if recombination also occurred between the $y^1$ locus (1A) and the $w^{1118}$ locus (3B), however as the $w^{1118}pcm^{14}P_{neoFRT}19A/FM7i$ flies were not yellow bodied, this was not the case.

In order to molecularly confirm the presence of the $pcm^{14}$ mutation in the $w^{1118}pcm^{14}P_{neoFRT}19A/FM7i$ stock created, PCR was performed on the genomic DNA extracted from the larvae and flies of this stock. As can be seen in Figure 4.3A, the $pcm^{14}$ mutation is indeed present in this stock.

To show that the lethality associated with the X chromosome of this stock was indeed caused by the $pcm^{14}$ mutation, the lethality was rescued by crossing $w^{1118}pcm^{14}P_{neoFRT}19A/FM7i$ virgin females to $In(1)FM7/T(1;Y)B92y^1y^+BS$ males, which contain a translocation of region 18A5-18D1 (contains the pacman locus) onto the Y chromosome (Figure 4.3B). The presence of round eyed (non-FM7i) males in the offspring of this cross is genetic evidence to show that the lethality stems from the region of the X chromosome which is translocated, which contains pacman.
Figure 4.3. Confirmation of the presence of the pcm\textsuperscript{14} mutation in the \textit{w\textsuperscript{1118} pcm\textsuperscript{14} P(neoFRT19A)/FM\textsuperscript{7}i} stock.

(A) PCR was carried out on the genomic DNA extracted from the larvae and adult flies from the \textit{w\textsuperscript{1118} pcm\textsuperscript{14} P(neoFRT19A)/FM\textsuperscript{7}i} stock to confirm the presence of the \textit{pcm\textsuperscript{14}} mutation. \textit{sfn22/pel3r} primers were used (Figure 2.1) which will only amplify a band if the \textit{pcm\textsuperscript{14}} mutation is present. \textit{pcm\textsuperscript{14}} and \textit{pcm\textsuperscript{WT}} larvae were used as controls.

(B) Diagrammatic representation of the cross carried out to confirm that the lethality on the X chromosome of the \textit{w\textsuperscript{1118} pcm\textsuperscript{14} P(neoFRT19A)/FM\textsuperscript{7}i} stock stems from the \textit{pacman} locus. Crossing \textit{w\textsuperscript{1118} pcm\textsuperscript{14} P(neoFRT19A)/FM\textsuperscript{7}i} virgin females to \textit{In(1) FM7/T(1;Y)B92 y\textsuperscript{i} y\textsuperscript{+} B\textsuperscript{5}} males, which contain a translocation of region 18A5-18D1 of the X chromosome (which contains the \textit{pacman} locus) onto the Y chromosome, rescued the lethality associated with hemizygous \textit{pcm\textsuperscript{14}} males.
4.4 Populations of *pcm*"14 wing imaginal disc cells have reduced growth when compared to populations of wild-type wing imaginal disc cells

In order to generate populations of *pcm*"14/*pcm*"14 and *pcm*"/*pcm*"14 cells in *pcm*"14/*pcm*"14 larvae, *w*"118 *pcm*"14 *P(neoFRT)19A/FM7* virgin females were crossed to *P(Ubi-mRFP.nls)1 w*" *P[hsFLP]12 P(neoFRT)19A* males to create *w*"118 *pcm*"14 *P(neoFRT)19A/P(Ubi-mRFP.nls)1 *w*" *P[hsFLP]12 P(neoFRT)19A* larvae (Figure 4.4A). These larvae are heterozygous for the *pcm*"14 mutation, contain a mRFP reporter on the wild-type chromosome distal to the *pacman* locus, contain the FRT sequence at position 19A on both chromosomes proximal to the *pacman* locus and also have the FLP under heat-shock control. These larvae were reared at 25°C and then placed in a vial in a water bath at 37°C for 1h to activate the FLP to induce recombination at the FRT site, either 24h or 48h after egg lay (AEL). Larvae were then placed back at 25°C until they reached the L3 stage of development and the discs were dissected and photographed with the confocal microscope.

If the *pcm*"14 mutation does result in a reduced growth rate, then the size of the mutant clones would be expected to be smaller when compared to their wild-type twin spots (Figure 4.1B). When mitotic recombination was induced 24 hours AEL only the wild-type twin spots were visible but no mutant clones were present (Figure 4.4B). This suggested that the wild-type cells had a large growth advantage over the mutant cells and that competition between the two populations of cells had prevented the mutant cells from forming a population (Neto-Silva et al, 2009). When mitotic recombination was induced 48 hours AEL, mutant clones are clearly visible alongside the wild-type twin spots, which were larger in size, showing that wild-type cells do indeed have a growth advantage over *pcm*"14 cells (Figure 4.4C).

This result therefore demonstrates a cell autonomous requirement of Pacman for the correct growth of the wing imaginal disc cells. This therefore further supports the hypothesis that the reason the wing imaginal discs are smaller in *pcm*"14 L3 larvae is because of a lack of functional Pacman in the wing imaginal disc cells themselves, as opposed to a lack of functional Pacman throughout the larvae which is causing the discs to arrest in growth due to global problems with development. The reasons behind this cell autonomous requirement of Pacman will be investigated below.
Figure 4.4. Populations of wild-type cells have a significant growth advantage over \textit{pcm}^{14} cells.

Mosaic analysis was performed to directly compare growth rates between wild-type and \textit{pcm}^{14} mutant cells. (A) Diagrammatic representation of the cross to generate \textit{w}^{1118} \textit{pcm}^{14} \textit{P(neoFRT)19A} \textit{P(Ubi-mRFP.nls)1} w^{*} \textit{P(hsFLP)12} \textit{P(neoFRT)19A} larvae. By selecting against GFP the \textit{w}^{1118} \textit{pcm}^{14} \textit{P(neoFRT)19A} chromosome is selected for. By selecting for RFP, the \textit{P(Ubi-mRFP.nls)1} w^{*} \textit{P(hsFLP)12} \textit{P(neoFRT)19A} is selected for. (B) When mitotic recombination is induced by heat-shock 24h AEL in \textit{w}^{1118} \textit{pcm}^{14} \textit{P(neoFRT)19A}\textit{P(Ubi-mRFP.nls)1} w^{*} \textit{P(hsFLP)12} \textit{P(neoFRT)19A} larvae, only the wild-type clones (arrow) are visible in the wing imaginal discs of the L3 larvae. (n=11) (C) When mitotic recombination is induced by heat-shock 48h AEL in \textit{w}^{1118} \textit{pcm}^{14} \textit{P(neoFRT)19A}\textit{P(Ubi-mRFP.nls)1} w^{*} \textit{P(hsFLP)12} \textit{P(neoFRT)19A} larvae, both the wild-type (arrow) and the mutant (arrow head) clones are clearly identifiable in the wing imaginal discs of the L3 larvae. In all wing imaginal discs observed, the wild-type clones were significantly larger than the mutant clones, demonstrating a growth advantage for wild-type cells over \textit{pcm}^{14} cells. (n=25). Background cells are \textit{pcm}^{WT}/\textit{pcm}^{14}, cells increased in fluorescence are \textit{pcm}^{WT}/\textit{pcm}^{WT} and cells with no fluorescence are \textit{pcm}^{14}/\textit{pcm}^{14}. Scale bars represent 100µm.
4.5 Loss of Pacman induces ectopic apoptosis in the wing pouch region of the wing imaginal discs

The smaller size of the $pcm^{14}$ wing imaginal discs could be due to an increase in apoptosis, a decrease in cell division, or a combination of both. In order to determine whether there was an increase in apoptosis in $pcm^{14}$ wing imaginal discs compared to wild-type, discs were stained with an anti-activated Caspase 3 antibody, which stains cells undergoing apoptosis. In the $pcm^{14}$ wing imaginal discs, large groups of cells in the wing pouch were undergoing apoptosis (Figure 4.5B). This does not occur in the wild-type discs (Figure 4.5A). Therefore loss of Pacman appears to induce apoptosis in the wing pouch region of the disc, which could account for the small size of the wing discs.

To determine whether loss of Pacman induces apoptosis only in the wing pouch, *pacman* was knocked down in different regions of the disc using the GAL4-UAS system and apoptosis was observed by staining the discs with the anti-activated Caspase 3 antibody. The cells where knockdown occurs were marked by GFP expression. As can be seen from Figure 4.6A'-A", knockdown of *pacman* over the entire disc using the 69B-GAL4 driver results in apoptosis only in the wing pouch region of the disc. As expected, knockdown of *pacman* specifically in the wing pouch, using the nub-GAL4 driver resulted in apoptosis throughout the wing pouch region of the disc (Figure 4.6B'). Furthermore, knockdown of *pacman* only in the posterior compartment of the disc with the eng-GAL4 driver, causes apoptosis to occur only in the posterior part of the wing pouch (Figure 4.6C'').
Figure 4.5. Ectopic apoptosis occurs throughout and specific to the wing pouch region of the wing imaginal discs in pcm^{14} L3 larvae. pcm^{WT} and pcm^{14} L3 wing imaginal discs were stained with anti activated Caspase 3 antibody, which marks cells undergoing apoptosis. Apoptosis was observed throughout and specific to the wing pouch of pcm^{14} wing imaginal discs (B), which is not observed in pcm^{WT} wing imaginal discs (A). (n≥39). Scale bars represent 100µm.
Figure 4.6. Knockdown of pacman using RNAi causes apoptosis specifically in the wing pouch region of the wing imaginal discs in L3 larvae. UAS-pcmRNAi was driven throughout the disc using the 69B-GAL4 driver (A-A’’), throughout the wing pouch using the nub-GAL4 driver (B-B’’) or throughout the posterior compartment of the disc using the eng-GAL4 driver (C-C’’). The wing imaginal discs were stained with anti-activated Caspase 3 antibody, demonstrating that apoptosis was occurring in cells specific to the wing pouch region of the disc where pacman was being knocked down. GFP fluorescence indicates regions of the disc expressing the UAS-pcmRNAi construct (n≥6). Note that these imaginal discs express UAS-GFP as well as UAS-pcmRNAi under the control of the relevant driver. Scale bars represent 100µm.
These experiments show that in the absence of Pacman in the wing imaginal disc cells, the cells undergo ectopic apoptosis during the larval stages of development, which could indeed explain the reduced growth and development of these discs and also the delayed development of the larvae. Whether or not this is a direct or indirect effect will be the subject of further experiments and will be discussed further in the discussion.

The fact that apoptosis is only occurring in the wing pouch region is perhaps surprising when Pacman has been shown in the previous chapter to be required throughout the disc (Figure 3.9D) and that it is expressed ubiquitously throughout the disc (Figure 3.4). Furthermore the whole disc is proportionally smaller, rather than only the wing pouch being smaller. One possible explanation could be that apoptosis is occurring throughout the disc at earlier stages of development and is only restricted to the wing pouch during the L3 stage. It is however very difficult to dissect wing imaginal discs from L2 larvae and this is made increasingly difficult by the fact that pcm14 wing imaginal discs are significantly smaller than wild-type. The fact that mutant clones were smaller than the wild-type twin spots throughout the disc and not just in the wing pouch region (Figure 4.4) supports the argument that apoptosis could be occurring throughout the disc earlier in development and is restricted to the wing pouch only during late L3.

### 4.6 The pcm14 mutation results in compensatory proliferation of wing imaginal disc cells

The reduced size of pcm14 wing imaginal discs as well as the results of the mosaic analysis experiment above, where pcm14/pcm14 mutant clones were smaller than pcm+/pcm+ clones, suggested that loss of Pacman in the pcm14 mutant imaginal disc cells could also result in a decrease in the rate of cell division. In order to test this, the rate of cell division in the pcm14 wing imaginal discs, compared to wild-type, was monitored by staining the discs with an anti-phosphohistone H3 antibody, which detects cells undergoing mitosis (Figure 4.7A and B). The nuclei undergoing division were counted and the mitotic index (the number of cells in M phase/area of disc) was calculated.

In order to count the number of cells in M phase objectively, the ImageJ plug-in termed DeadEasy MitoGlia was used (Forero et al, 2010). This program has been specifically designed to count cells labelled with phosphohistone H3 in Drosophila embryos, although it can also be used for tissues such as imaginal discs. Once the settings were optimised set so that the amount of false positives and false negatives were reduced as much as possible
(see 2.7.4), these were kept the same and all the conditions of the staining and microscopy were also kept identical and performed at the same time. Z-stack images taken on the confocal microscope were then imported into ImageJ and the number of cells in M phase was calculated. Figure 4.7 A’ and B’ show the images exported from the DeadEasy MitoGlia plug-in, with each white dot representing a positive count for a cell in M phase.

This experiment showed that the rate of cell division in mutant discs is actually 51% higher than in wild-type discs (Figure 4.7C). It would therefore appear that the cells within mutant discs are undergoing compensatory proliferation in an attempt to overcome the increased rate of apoptosis (Fan & Bergmann, 2008a; Martin et al, 2009). This could also explain the diffuse expression of Wingless in the wing pouch region of pcm14 discs (Figure 3.15B) as cells undergoing apoptosis have been shown to secrete Wingless in order to induce compensatory proliferation in neighbouring cells (Huh et al, 2004; Perez-Garijo et al, 2004; Ryoo et al, 2004). Nevertheless, the smaller size of the mutant discs, even after the extended period of larval development, means that this compensatory proliferation is unable to counter the increased levels of apoptosis.

The above experiments support the hypothesis that it is an increase in apoptosis that is causing the reduced growth of the wing imaginal discs. Furthermore, there is no reduction in cell division, which shows that the apoptosis is the sole contributor to this phenotype. However, this is based on the assumption that cell size is not affected by the pcm14 mutation. This caveat was attempted to be addressed by staining the discs with DAPI to label individual nuclei so that total cell number could be counted. However, this proved to be difficult due to the small size of the cells and also the structure of the disc. As the disc is made up of two layers of cells and contains several folds, it was difficult to gain the resolution to accurately count the number of cells within the disc.
Figure 4.7. The mitotic index is greater in pcm\textsuperscript{14} wing imaginal discs compared to wild-type.

(A) pcm\textsuperscript{WT} and (B) pcm\textsuperscript{14} L3 wing imaginal discs were stained with an anti-phosphohistone H3 antibody, which labels cells in M phase. Scale bars represent 100\(\mu\)m. (\(A'\) and \(B'\)) represent the images of A and B which have been exported from the DeadEasy MitoGlia plug in. Each white dot represents a positive count for a cell in M phase. (C) A mitotic index was calculated by dividing the number of cells in M phase by the area of the disc. The mitotic index was increased in pcm\textsuperscript{14} compared with wild-type (\(n\geq 14\), \(p<0.001\). Error bars represent 95\% confidence limits.)
4.7 Chapter summary

The main conclusions from the experiments in this chapter is that wing imaginal disc cells have a cell autonomous requirement for Pacman for their correct growth and development. This is demonstrated by the observation that populations of wild-type cells can out compete populations of pcm14 cells and form larger clones. This is caused by an increase in apoptosis in pcm14 cells. It also appears that as a result of the apoptosis, compensatory proliferation is occurring in an attempt to counteract the apoptosis, but that this is clearly not enough to prevent the reduced growth of the discs.

4.8 Chapter discussion

4.8.1 Why is apoptosis restricted to the wing pouch of the discs?

It is perhaps surprising that the apoptosis in pcm14 wing imaginal discs is restricted to the wing pouch of the discs, considering that the whole disc is proportionally smaller and that Pacman has been shown to be both expressed (Figure 3.4) and required (Figure 3.9) throughout the disc. Furthermore, the mosaic analysis experiment shows that the cell autonomous requirement of Pacman is not dependent on the position of the cells within the disc, as all mutant clones are smaller than their wild-type twin spots (Figure 4.4). It is very likely that apoptosis is occurring in the wing imaginal discs earlier in development as the discs are already significantly smaller in the L3 stage of development. Therefore the most likely explanation is that apoptosis is occurring throughout the disc earlier in development and is restricted to the wing pouch only during late L3. However, as mentioned this is hard to test as wing imaginal discs are very difficult to dissect in the L2 stage and this is made increasingly difficult by the reduced size of pcm14 wing imaginal discs.

The reason that apoptosis is occurring specifically within the wing pouch during the L3 stage of development is unclear. The most likely explanation depends on whether Pacman is regulating apoptosis directly or indirectly. If Pacman is directly regulating apoptosis by selectively degrading certain pro-apoptotic mRNAs, then perhaps those mRNAs are only transcribed in the wing pouch region of the disc during L3. This could be tested by in situ hybridisation, but obviously such targets of Pacman would have to first be identified.
If *pcm*14 cells are undergoing apoptosis as a result of an indirect effect of the loss of Pacman, then the reason that apoptosis is restricted to the cells in the wing pouch region of the disc could be due to an increased selective pressure on these cells, which could be what tilts these cells into apoptosis but not the cells in the thorax region. The cells of the wing pouch are first determined during the L2 stage of development by the expression of Vestigial across the DV boundary (Couso et al, 1995). The number of cells expressing vestigial in the disc then greatly increases to give rise to the wing pouch of the disc and so the rate of proliferation is expected to be greater in the wing pouch cells of the disc. This could therefore explain the increased sensitivity of the wings to apoptotic stimuli and indeed it has been observed that in response to ionizing radiation, the wings are often the first tissues to give rise to apoptotic cells (personal communication with Juan Pablo Couso).

4.8.2 Why is the compensatory proliferation not sufficient to counteract the increased apoptosis?

Compensatory proliferation is a well-studied phenomenon in the development of multicellular organisms and was first discovered as early as 1977 (Haynie & Bryant, 1977). Apoptosis-induced compensatory proliferation is required to maintain tissue homeostasis so that damaged tissues can be replaced allowing the organ to maintain its normal size (Fan & Bergmann, 2008a; Martin et al, 2009). In proliferating tissue such as the wing imaginal discs this occurs via the initiator caspase Dronc (Huh et al, 2004; Kondo et al, 2006), which signals to neighbouring cells to proliferate through both the JNK and p53 signalling pathways and the morphogens Wingless and Dpp (Bergantinos et al, 2010; Huh et al, 2004; Perez-Garijo et al, 2009; Ryoo et al, 2004; Wells et al, 2006).

During these initial experiments it was shown that the apoptosis causes reduced growth and delayed development of the discs and larvae, but that the overall size of the adult remains unaffected. This raises the question as to why compensatory proliferation is not able to rescue the size of the discs in the *pcm*14 mutant. This failure of the wing discs to regenerate could be explained by the fact that there is likely prolonged apoptosis occurring in the *pcm*14 wing imaginal discs, whereas other experiments have induced a pulse of apoptosis, allowing time for the wing disc to recover (Perez-Garijo et al, 2004). Therefore the compensatory proliferation never has a chance to regain control of the size of the developing organ, as the cells that are induced to proliferate in response to the apoptosis rapidly undergo apoptosis themselves.
This would support the hypothesis in Chapter 3.16.3 that the reason that 69B-GAL4/UAS-\textit{pcm}^{RNAi} wing imaginal discs are not significantly smaller than their parental controls is that compensatory proliferation is preventing the apoptosis from reducing the size of the discs. The reason this is able to occur in discs in which \textit{pacman} has been knocked down using RNAi as opposed to knocked out with a null mutation is perhaps that in RNAi discs there is more of a balance between apoptosis and compensatory proliferation compared to the mutant discs. This could be because there is still low levels of Pacman protein in the RNAi discs, whereas in the mutant discs Pacman activity is completely lost.

The reason that the wing size is smaller in flies in which \textit{pacman} has been knocked down with RNAi is perhaps because proliferation rates slow during late larval stages and during pupation these cells only go through two more rounds of cell division before they begin differentiating into wings (Neto-Silva et al, 2009). Therefore there may be a window in development in which cells are still undergoing apoptosis but have lost the potential to replace these dying cells by compensatory proliferation, causing loss of tissue and the size of the wings to be smaller.

It is interesting to note the importance in understanding the mechanisms of compensatory proliferation. In order for the signalling pathways involved in compensatory proliferation to be elucidated, cells which were in the process of undergoing apoptosis had to be kept in an “un dead” state. To this end the authors made use of the Baculovirus caspase inhibitor p35, which inhibits the effector caspases but not the initiator caspases (Clem et al, 1991; Crook et al, 1993; Goyal, 2001; Hay et al, 1994). Therefore, p35 expression in cells in which the apoptosis pathway has been activated, will inhibit cell death but not compensatory proliferation. This therefore causes overgrowth of the tissue and developmental aberrations (Perez-Garijo et al, 2004; Perez-Garijo et al, 2009). This therefore has implications for cancer treatment. A common cancer treatment in cancer therapy is to attempt to cause the cancer cells to undergo apoptosis. If however the cancer cells have lost the ability to induce apoptosis due to a mutation in the effector caspases, then activating the apoptosis pathway would cause an increase in growth due to the induction of compensatory proliferation, causing increased growth of the tumour (Fan & Bergmann, 2008b).

\textbf{4.8.3 Why is the compensatory proliferation not concentrated to cells neighbouring the apoptotic cells?}
One might expect that if compensatory proliferation were happening, it would occur near the region of apoptosis. However, the pattern of cells in M phase in Figure 4.7 show that the increased proliferation in pcm14 wing imaginal discs is throughout the whole disc and not concentrated around the wing pouch. There are two possible explanations for this.

Firstly, as discussed above, although the apoptosis is restricted to the wing pouch in late L3 discs, this may not be the case earlier in development. Therefore if apoptosis was occurring throughout the disc then the compensatory proliferation would also be expected to occur throughout the disc.

Also, even if the apoptosis is restricted to the wing pouch throughout development, it is clear that the whole disc is still proportionally smaller. Therefore compensatory proliferation would need to occur throughout the disc in order to reach a critical size for pupariation to occur.

Although it has been shown that compensatory proliferation occurs in neighbouring cells to those undergoing apoptosis, these experiments have been carried out in undead cells using the caspase inhibitor p35 (Perez-Garijo et al, 2004). Therefore in these experiments the cells destined for apoptosis are kept alive and continue to signal to neighbouring cells to proliferate. Therefore, the concentration of proliferative morphogens will always be higher surrounding the "undead cells". It is known that apoptotic cells can carry out long range signalling in the developing wing imaginal discs (Perez-Garijo et al, 2013). It could therefore be that in pcm14 wing imaginal discs, the apoptotic cells are signalling throughout the disc for all cells to increase the rate of proliferation.

4.8.4 Is Caspase 3 staining a reliable indication of apoptotic cells?

The activated Caspase 3 antibody used in this study is commonly used in apoptosis research in Drosophila, despite the antibody being raised to cleaved human Caspase 3. It has been shown to overlap with other markers of cell death such as TUNEL staining and so it was presumed that it detects the Drosophila homologues of Caspase 3, Dr-ICE and DCP-1. However, as reported in Fan and Bergmann 2010, immunoreactivity of this antibody persists in double mutants of Dr-ICE and DCP-1. They provide evidence that although the antibody does bind DCP-1 and likely Dr-ICE, it also binds at least one other protein, which is downstream of Dronc in the apoptosis pathway (Fan & Bergmann, 2010). This unknown
protein could be involved in apoptotic or non-apoptotic signalling and so could be problematic for some studies.

Although this antibody could therefore be binding to additional substrates of Dronc, it is unlikely that it is not also binding either Dr-ICE or DCP-1 or both as the phenotypes observed are consistent with an increase in apoptosis in these discs. To ensure this, the following chapter will use genetics to confirm that the reduced growth of pcm\textsuperscript{14} discs are indeed a direct consequence of an increase in apoptosis.
5 How is Pacman regulating apoptosis in the wing imaginal discs?

5.1 Introduction

In the previous chapter it was shown that wing imaginal disc cells have a cell autonomous requirement for Pacman in order to prevent the cells from undergoing apoptosis. However, the experiments carried out were unable to determine whether the reduced growth and delayed development of the *pcm*¹⁴ wing imaginal discs were a direct consequence of this apoptosis and whether Pacman was directly or indirectly regulating apoptosis. Addressing these questions will therefore be the focus of this chapter.

5.1.1 Programmed cell death (Apoptosis)

Apoptosis, or programmed cell death, is crucial for the development of multicellular organisms, as well as being important in disease. The key components of apoptosis pathways are well known and highly conserved, and many of the signalling pathways that regulate apoptosis have been elucidated (Danial & Korsmeyer, 2004; Fuchs & Steller, 2011; Hay & Guo, 2006; Salvesen & Abrams, 2004; Xu et al, 2009). The regulation of apoptosis involves a wide range of intra and extra-cellular stimuli, including developmental signals and cellular stresses such as DNA damage, unfolded protein response, ER stress, reactive oxygen species and defects in cell specification/differentiation (Steller, 2008). The apoptotic events are carried out by a set of cysteine proteases named caspases. There are two main pathways in which apoptosis is activated (Danial & Korsmeyer, 2004). The extrinsic pathway signals through the cell surface death receptors such as Fas and TRAIL. This leads to the activation of the Caspase 8 and is particularly important in the immune response (Debatin & Krammer, 2004). The intrinsic pathway responds to signals within the cell, such as developmental cues or DNA damage and involves the activation of the apoptosome. In mammalian cells this involves cytochrome c release from the mitochondria (Jiang & Wang, 2004), whereas in *Drosophila* this involves releasing the inhibitory effect imposed by the *Drosophila* Inhibitor of Apoptosis Protein (DIAP1) (Hay & Guo, 2006; Salvesen & Abrams, 2004; Steller, 2008; Xu et al, 2009). Both these pathways converge at the level of effector caspase activation, which represents the critical step in the induction of apoptosis, after which the cell is destined to undergo suicide (Figure 5.1) (Danial & Korsmeyer, 2004; Hay & Guo, 2006; Salvesen & Abrams, 2004). The cellular processes required for apoptosis to occur includes the condensation of the nucleus and cytoplasm,
the degradation of cellular proteins and DNA, global inhibition of translation and the fragmentation of the apoptotic cells into membrane bound bodies which are rapidly phagocytosed by neighboring cells (Domingos & Steller, 2007; Thomas & Lieberman, 2013). Apoptosis is therefore a highly controlled process required to remove damaged or unwanted cells in a regulated manner so as not to cause further harm to the organism. It is therefore distinct from necrosis, which results from traumatic injuries and causes the cells to swell and lyse, releasing their cytoplasmic contents into the extracellular space. Autophagy is also a mechanism of cell death. Interestingly, autophagy evolved as a survival mechanism in unicellular organisms deprived of nutrients in which the cytoplasmic contents are engulfed by autophagosomes and are then degraded and recycled within the lysosome (Benbrook & Long, 2012). However, it appears that during metazoan evolution autophagy has developed as an alternative mechanism for cell death (Kourtis & Tavernarakis, 2009; McPhee & Baehrecke, 2009; Ryoo & Baehrecke, 2010). The relationship between cell death and life seems complex (Baehrecke, 2005) and the relationship between autophagy and apoptosis is not clear. However, autophagy has been shown to be involved in the removal of the tissues during development, such as the mid-gut during *Drosophila* pupation (Denton et al, 2009).

The regulation of apoptosis during development and in response to stress has been extensively studied in *C. elegans, Drosophila* and mice. These studies have highlighted the high conservation of these apoptosis pathways and have therefore been instrumental in understanding the genetic regulation of apoptosis in humans. Although there are differences between the pathways between these species, they all initiate apoptosis through the activation of specific cysteine proteases named caspases (Kumar, 2007). Caspases are expressed as inactive zymogens in almost all, if not all, cells and are split into two families named initiator (apical) caspases and effector (executioner) caspases. These caspases contain a small and large subunit which are cleaved to form an active tetramer composed of two small and two large subunits. They also contain a prodomain at the N terminal which is also cleaved during activation. This prodomain is much larger in the initiator caspases and contains protein interaction domains such as the Caspase Recruitment Domain (CARD) found in human Caspase 9 and the *Drosophila* and *C. elegans* homologues Dronc and CED-3 respectively (Kumar, 2007). The final step in the initiation of apoptosis is the cleavage of the effector caspases which triggers a cascade of downstream events resulting in cell death. As these effector caspases are expressed as inactive zymogens in almost all cells, it is no surprise that mammals, *Drosophila* and *C. elegans* have
evolved distinct yet overlapping mechanisms in which to regulate the activation of these caspases (Salvesen & Abrams, 2004). The apoptosis occurring in pcm14 wing imaginal discs is very cell specific and seems to be developmentally regulated. It is therefore most likely being activated by the intrinsic pathway which will be discussed in detail below. An overview of the intrinsic apoptosis pathway in Drosophila and mammals is shown in Figure 5.1.
Figure 5.1. Diagrammatic representation of the intrinsic cell death pathways in *Drosophila* and mammals.

(A) In *Drosophila*, apoptosis is initiated by the IAP antagonists, of which the three most extensively studied are Hid, Reaper and Grim. These proteins inhibit the *Drosophila* inhibitor of apoptosis protein (DIAP1), which under normal conditions inhibits the initiator caspase Dronc. Activated Dronc can then bind the adapter protein Ark (Apaf1 Related Killer) to form the apoptosome, which then cleaves and activates the effector caspases Dr-ICE and Dcp1. These effector caspases then initiate the downstream events that cause the cell to undergo apoptosis.  

(B) In mammals the main input into the apoptosis pathway is through the action of the Bcl-2 family of proteins, which possess either pro- or anti-apoptotic activity. Mammals also possess inhibitor of apoptosis proteins, such as X-linked IAP (XIAP), which is also regulated by IAP antagonists. However, it appears that apoptosis is predominantly regulated in mammals by the release of cytochrome C from the mitochondria, which stimulates binding of Caspase 9 by Apaf-1 to form the apoptosome. This then cleaves downstream effector caspases to trigger apoptosis.
5.1.2 Intrinsic apoptosis pathway in Drosophila

Apoptosome and the effector caspases

The Drosophila genome encodes seven caspases (Kumar, 2007). Of these, only Dronc, a Caspase-9-like initiator caspase (Doryst et al., 1999), and the Caspase-3-like effector caspases Dr-ICE (Fraser & Evan, 1997; Fraser et al., 1997) and Dcp-1 (Song et al., 1997) have been shown to be crucial for the majority of developmental and stress induced apoptosis. Dronc is the only caspase to possess a Caspase Recruitment Domain (CARD), with which it interacts with the adapter protein Ark (Apaf-1-related killer) (also known as Dark, D-Apaf-1 and Hac-1) to form the apoptosome (Doryst et al., 1999; Quinn et al., 2000; Xu et al., 2005). In agreement with Dronc being the only initiator caspase in Drosophila, Dronc activity has been shown to be essential for almost all cell death during development and in response to stress (Chew et al., 2004; Daish et al., 2004; Mills et al., 2005; Walshuber et al., 2005; Xu et al., 2005).

Ark has been shown to be the functional homologue of Apaf-1 (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999) and is required for apoptosome activity by interacting with Dronc via its own CARD. Ark has been shown to be essential for almost all programmed and stress induced cell death (Akdemir et al., 2006; Mills et al., 2006; Srivastava et al., 2007; Zimmermann et al., 2002).

The two most important effector caspases in the Drosophila apoptosis pathway are Dr-ICE and Dcp-1, both of which share homology with mammalian Caspase 3 (Fraser & Evan, 1997; Fraser et al., 1997; Song et al., 1997). Double mutants for Dr-ICE and Dcp-1 blocks almost all embryonic cell death and cause pupal lethality, yet single mutants only show a slight reduction in apoptosis and are viable as adults (Xu et al., 2006).

DIAP1

In order to prevent apoptosome formation in non-apoptotic cells, Dronc and Dr-ICE are repressed through a direct interaction with Drosophila Inhibitor of Apoptosis Protein 1 (DIAP1) (Ditzel et al., 2008; Hay et al., 1995; Meier et al., 2000b). DIAP1 is part of a family of IAPs conserved from humans to yeast (O'Riordain et al., 2008). The interaction between DIAP1 and Dronc and Dr-ICE is mediated by its Baculovirus Inhibitory Repeat (BIR) domains, of which all IAPs contain between one and three (O'Riordain et al., 2008; Salvesen & Duckett, 2002; Steller, 2008; Xu et al., 2009). Some IAPs, including DIAP1 and the human homologue XIAP also contain a RING domain, conferring E3-ubiquitin ligase activity to the protein (O'Riordain et al., 2008; Salvesen & Duckett, 2002).
DIAP1 is the main IAP in *Drosophila* (also named Thread) and its importance is highlighted by studies in which DIAP1 has been knocked out, causing massive widespread apoptosis (Goyal et al, 2000; Lisi et al, 2000; Wang et al, 1999). In non-apoptotic cells DIAP1 binds both Dronc and Dr-ICE through its BIR domains and inhibits their activity through ubiquitination by its RING domain (Ditzel et al, 2008; Wilson et al, 2002). In apoptotic conditions, the pro-apoptotic RHG (Reaper, Hid and Grim) proteins stimulate autoubiquitination of DIAP1 which mediates its degradation by the proteosome (Holley et al, 2002; Ryoo et al, 2002; Wilson et al, 2002; Yoo et al, 2002).

**RHG proteins (IAP antagonists)**

In order to induce apoptosis in *Drosophila* cells the inhibitory effect of DIAP1 on Dronc and Dr-ICE needs to be alleviated. This is achieved through the actions of the RHG (Reaper, Hid and Grim) proteins (Zachariou et al, 2003). The genes of these three proteins are located very close to each other in the genome and were the first pro-apoptotic genes to be discovered in *Drosophila* in a deficiency screen which generated the *Df(3L)H99* deletion (Chen et al, 1996; Grether et al, 1995; White et al, 1994). Homozygous *Df(3L)H99* embryos lack almost all developmental cell death and most irradiation-induced cell death. The proteins have no catalytic activity and share little similarity, except that they all contain a short IAP Binding Motif (IBM) (also termed RHG motif) (Wing et al, 2001) which binds the BIR domains of DIAP1 (Goyal et al, 2000; Zachariou et al, 2003). This interaction competes with the interaction between the BIR domain of DIAP1 and the caspases Dronc and Dr-ICE and also stimulates DIAP1 autoubiquitination and degradation (Holley et al, 2002; Ryoo et al, 2002; Wilson et al, 2002; Yoo et al, 2002). Although these genes appear to act synergistically in certain cell types (Peterson et al, 2002; Robinow et al, 1997; Wing et al, 1998; Xu et al, 2009; Yu et al, 2002; Zhou et al, 1997), expression of each gene is sufficient to induce apoptosis both in cells which normally live (Nordstrom et al, 1996; White et al, 1996) and in *Df(3L)H99* embryos (Grether et al, 1995; Hay et al, 1995; White et al, 1994).

Since these initial discoveries, three additional RHG proteins have been discovered, named *sickle* (Christich et al, 2002; Srinivasula et al, 2002; Wing et al, 2002), *jafrac2* (Tenev et al, 2002) and *dOmi/HtrA2* (Challa et al, 2007; Igaki et al, 2007; Khan et al, 2008).
5.1.3 Conservation of the apoptosis pathways in *Drosophila* and mammals

The role of the mitochondria and cytochrome c

The main difference in the regulation of caspase activity between mammals and *Drosophila* is that apopotosome formation in mammalian cells requires the release of cytochrome c from the mitochondria into the cytoplasm during mitochondrial outer membrane permeabilisation (MOMP) (Danial & Korsmeyer, 2004; Jiang & Wang, 2004; Kroemer et al, 2007). This is regulated primarily by the Bcl-2 family of proteins which can have either pro- or anti-apoptotic effects (Youle & Strasser, 2008).

The role of the MOMP and cytochrome c for the induction of apoptosis in *Drosophila* is not clear. Although *Drosophila* possesses homologs of cytochrome c, these have been shown not to be required for apopotosome formation and apoptosis (Dorstyn et al, 2004; Dorstyn et al, 2002; Yuan et al, 2011). However, there have also been studies in which MOMP was observed during apoptosis (Abdelwahid et al, 2007; Goyal et al, 2007) and cytochrome c has been shown to be required for an apoptosis-like process during sperm differentiation (Arama et al, 2003; Arama et al, 2005). Interestingly the RHG proteins in *Drosophila* have been shown to localise to the outer membrane of the mitochondria in order to efficiently inhibit apoptosis (Claveria et al, 1998; Freel et al, 2008; Haining et al, 1999; Olson et al, 2003; Sandu et al, 2010).

IAPs

The human genome encodes eight known IAPs, of which the most extensively studied is the X-linked IAP (XIAP) (O’Riordan et al, 2008). Similar to DIAP1, XIAP is able to block both the initiator Caspase 9 and the effector Caspases 3 and 7 (Deveraux et al, 1997; Shiozaki et al, 2003; Takahashi et al, 1998). Although it was initially thought that other IAPs also inhibited caspase activity, it appears that this is not the case (Eckelman et al, 2006). It is perhaps surprising therefore that XIAP null mice do not display any obvious cell death phenotype (Harlin et al, 2001; Olayioye et al, 2005). However it is possible that there is functional redundancy with other IAPs, which could inhibit apoptosis via alternative mechanisms (Eckelman et al, 2006; O’Riordan et al, 2008). Interestingly, IAP overexpression has been shown to contribute to apoptotic resistance in cancer cells and IAP antagonists are being developed as cancer therapeutics (Dynek & Vucic, 2013; Hunter et al, 2007).
IAP antagonists in mammals

Based on the sequence of the RHG proteins in *Drosophila* no obvious homologs of these genes were initially identified in the mammalian genome. However, expression of *reaper*, *hid*, and *grim* is sufficient to induce apoptosis in mammalian cells (Clavería et al, 1998; Haining et al, 1999; McCarthy & Dixit, 1998), suggesting that the mechanism of IAP antagonism is conserved. Immunoprecipitation of XIAP identified two IAP antagonists named HtrA2 (Omi) (Hegde et al, 2002; Martins et al, 2002; Suzuki et al, 2001; van Loo et al, 2002; Verhagen et al, 2002) and Smac (Diablo) (Du et al, 2000; Ekert et al, 2001; Verhagen et al, 2000). Unlike the *Drosophila* RHG proteins, Smac and HtrA2 are ubiquitously expressed and localised to the inner membrane of the mitochondria in non-apoptotic cells and are released into the cytoplasm by MOMP during apoptosis. Interestingly, Smac and HtrA2-deficient mice have no apoptosis phenotypes (Martins et al, 2004; Okada et al, 2002). An additional IAP antagonist has been identified more recently, named ARTS. Unlike Smac and HtrA2, ARTS is localised to the mitochondria outer membrane, is the only known IAP antagonist not to possess an IBM domain and has been shown to act upstream of cytochrome c (Edison et al, 2012; Gottfried et al, 2004; Larisch et al, 2000). Also ARTS has been shown to act as a tumour suppressor as mice depleted of ARTS have increased tumour development (García-Fernández et al, 2010).

5.1.4 Releasing the brakes or stepping on the gas?

The best coined phase to describe the differences between the apoptosis pathways in mammals and *Drosophila* is that *Drosophila* act by releasing the brakes whereas mammals act by stepping on the gas (Salvesen & Abrams, 2004). Although it appears that the existence of the mitochondria pathway predates the emergence of vertebrates (Bender et al, 2012), the role of MOMP in *Drosophila* has remained controversial. Furthermore although the role of IAPs and IAP antagonism is still functionally present in mammals, the importance of this pathway is not clear and is masked by functional redundancy. It therefore appears that *Drosophila* and mammals have evolved to make use of alternative yet overlapping pathways in order to prevent unwanted caspase activation and cell death.

5.1.5 Regulation of apoptosis in *Drosophila*

Historically, it has been perceived that activation of the apoptosis pathway in *Drosophila* is through the transcriptional activation of the genes encoding the RHG proteins (Steller, 2008; Xu et al, 2009). Indeed, the accumulation of *reaper* and *grim* mRNAs are observed in
neurons destined to undergo cell death (Robinow et al, 1997) and these genes are under the control of a common regulatory region (Tan et al, 2011). Several transcription factors have been shown to regulate these RHG genes, including p53 (Brodsky et al, 2000), RBF (Tanaka-Matakatsu et al, 2009), the Hox gene Deformed (Lohmann et al, 2002) and the ecdysone EcR/USP complex (Jiang et al, 2000). Epigenetic regulation has also been shown to occur to block reaper and hid transcription in response to irradiation in post stage 12 embryos. This occurs through the trimethylation of H3K27/H3K9 within the irradiation-responsive enhancer region (IRER) which controls both these genes (Zhang et al, 2008). In addition to transcriptional regulation, the activity of Hid can be regulated post-translationally by the pro-survival EGF-receptor/Ras-pathway (Bergmann et al, 1998; Bergmann et al, 2002). Translational inhibition of hid mRNA was also shown to occur in the wing imaginal disc cells by the miRNA bantam (Brennecke et al, 2003).

Although post-transcriptional processes that work at the level of RNA stability are known to be important in a number of cellular processes, such as inflammation (Sanduja et al, 2011), their contribution in the control of apoptosis is not fully appreciated. However there is growing evidence of a network of RNA binding proteins and miRNAs that regulate the stability of these pro-apoptotic mRNAs post-transcriptionally. For example, many miRNAs have been shown to regulate apoptosis in Drosophila (Bejarano et al, 2010; Brennecke et al, 2003; Ge et al, 2012; Hilgers et al, 2010; Jovanovic & Hengartner, 2006; Karres et al, 2007; Leaman et al, 2005; Xu et al, 2004; Xu et al, 2003; Zhang & Cohen, 2013). It is not clear however whether these miRNAs are functioning at the level of mRNA degradation or translational inhibition. For example, the first miRNA shown to regulate apoptosis was bantam which was shown to bind to the 3'UTR of hid and inhibit translation. However, levels of hid mRNA were not shown to be effected (Brennecke et al, 2003). Despite this, Dcp1 and Dcp2 were shown to be required for the repression of a hid 3'UTR reporter construct in the eye imaginal disc (Pressman et al, 2012), suggesting that additional factors could be regulating hid mRNA stability. A role for RNA binding proteins has also been identified, in that the RNA-binding protein HuR (homologue of Elav in Drosophila) has recently been shown to be cleaved in HeLa cells during caspase-mediated apoptosis with the two cleavage fragments binding to and stabilising Caspase 9 mRNA, thus promoting apoptosis (von Roretz et al, 2013). Evidence to support the general importance of RNA stability for the regulation of apoptosis comes from work on the deadenylases Ccr4a and Ccr4b which can affect cell survival in MCF7 human breast cancer cells (Mittal et al, 2011).
5.2 Aims

The aims of this chapter are to determine whether Pacman directly regulates apoptosis, or whether the increase in apoptosis in pcm14 wing imaginal discs is caused by an indirect effect. The first hypothesis is that Pacman could be directly regulating the apoptosis pathway, by degrading certain pro-apoptotic mRNAs. Although mechanisms of post-transcriptional control of apoptosis have been reported, this would be the first time an exoribonuclease has been shown to directly regulate pro-apoptotic mRNA stability. It would therefore be an interesting and important observation to the field of apoptosis and cancer if Pacman was indeed regulating apoptosis directly.

The alternative hypothesis is that the loss of 5'-3' cytoplasmic degradation in the cells has caused the cells to be under stress conditions, which has led to the induction of apoptosis in these cells. Indeed, loss of essential genes often activates stress response pathways that transcriptionally induce pro-apoptotic genes. However, the fact that pcm14 mutants survive until pupation and the tissue specific nature of the apoptosis raises the question as to why do some cells survive and other cells undergo apoptosis. Also mis-specified cells undergo apoptosis during development in response to transcriptional up-regulation of hid (Werz et al, 2005). Therefore, if Pacman is involved in the specification of wing imaginal disc cells, then apoptosis in pcm14 wing discs could result from problems with cell specification.

In order to test the simplest hypothesis first, this chapter will address the hypothesis that Pacman is directly regulating apoptosis. This will be addressed by answering the following questions;

- Is apoptosis directly causing the reduced growth and delayed development of the wing imaginal discs?
- Are any pro-apoptotic genes up-regulated in pcm14 wing imaginal disc cells?
- If so, is the level of up-regulation transcriptional or post-transcriptional?
5.3 Inhibition of apoptosis partially rescues the reduced growth of pcm\textsuperscript{14} wing imaginal disc cells

In order to test directly whether the apoptosis observed in pcm\textsuperscript{14} wing imaginal discs was the cause of the reduced size of these discs, wing disc size was measured in pcm\textsuperscript{14} L3 larvae in which the apoptosis pathway had been inhibited. The apoptosis pathway in Drosophila is depicted in Figure 5.1. The genes encoding the pro-apoptotic RHG proteins are located adjacent to each other on the chromosome arm 3L and are removed by the deficiency Df(3L)H99 (Chen et al, 1996; Grether et al, 1995; White et al, 1994). In order to inhibit apoptosis, the Df(3L)H99 deletion was first crossed into the pcm\textsuperscript{14} mutant as a heterozygote (Figure 5.2A). pcm\textsuperscript{14};Df(3L)H99/+ discs were stained with the activated Caspase 3 antibody to determine whether levels of apoptosis had been reduced. Figure 5.3C shows that apoptosis in pcm\textsuperscript{14};Df(3L)H99/+ imaginal discs is markedly reduced compared to pcm\textsuperscript{14} discs (Figure 5.3B). This therefore shows that the apoptosis occurring in pcm\textsuperscript{14} discs is indeed induced through the intrinsic pathway shown in Figure 5.1. Figure 5.3D shows that the mean size of these discs was partially rescued to 81\% of the size for pcm\textsuperscript{14} discs. This is therefore a 65\% rescue in size ((81-45)/(100-45)*100). In accordance with there being low levels of apoptosis in wild-type wing imaginal discs development, with more extensive apoptosis occurring during the two moult periods (Milan et al, 1997), disc size was increased by 5\% in wild-type L3 larvae with the Df(3L)H99 deletion as a heterozygote (Figure 5.3D).

It has been shown that the Drosophila homologue of Apaf1, named Ark, is required for the formation of the apoptosome and is essential for almost all programmed cell death in Drosophila (Akdemir et al, 2006; Mills et al, 2006; Srivastava et al, 2007). As null mutations in Ark survive to the pupal stage of development (Akdemir et al, 2006), wing imaginal discs size was measured in wild-type and pcm\textsuperscript{14} discs homozygous for the null Ark\textsuperscript{82} mutation (Figure 5.2B). This significantly rescued the size of pcm\textsuperscript{14} wing imaginal discs to 85\% that of wild-type (Figure 5.3D), which is a 73\% rescue in size ((85-45)/(100-45)*100). This demonstrates that Ark is required for most but not all the ectopic apoptosis occurring in pcm\textsuperscript{14} wing imaginal discs. Wild-type wing imaginal disc size increased by 12\% in a homozygous Ark\textsuperscript{82} background (Figure 5.3D).
Figure 5.2. Diagrammatic representation of the crosses carried out in order to inhibit apoptosis in pcm14 L3 larvae.

(A) \( y^1 \) pcm14/FM7i/Y virgin females were crossed to \( w^+/Y ; Df(3L)H99\ knirp^1\ p^p/TM6B\ Tb\ Hu \) males in order to generate \( y^1 \) pcm14/Y;; Df(3L)H99 knirp^1 p^p/+ larvae. These larvae were selected from other genotypes in the offspring of this cross by selecting against the GFP present in the FM7i balancer chromosome, against the Tubby (Tb) marker on the TM6B Tb Hu balancer chromosome and for the recessive \( y^1 \) marker present on the \( y^1 \) pcm14 chromosome, which causes the mouth parts of the larvae to be brown in colour. (B) In order to generate \( y^1 \) pcm14/Y; ArkE2 larvae, \( y^1 \) pcm14/FM7i; ArkE2/CyO-GFP virgin females were crossed to FM7i/Y; ArkE2/CyO-GFP males and larva were selected for by selecting against the GFP present in the FM7i and CyO-GFP balancer chromosomes. (C) In order to generate \( y^1 \) pcm14/Y; GAL80^69B-GAL4/L4DIAP1 larvae \( y^1 \) pcm14/FM7i;; UAS-DIAP1 virgin females were crossed to \( w^*/; GAL80^69B-GAL4\ males and non-GFP larvae with brown mouth parts were selected for.
Figure 5.3. Inhibiting apoptosis partially rescues the size of the pcm^14 wing imaginal discs.

(A-C) Crossing the Df(3L)H99 deletion (which deletes the pro-apoptotic genes *hid*, *grim* and *reaper*) into *pcm*^14^ as a heterozygote inhibits apoptosis, as determined by the reduced Caspase 3 staining and increased size of the *pcm*^14^; *Df(3L)H99* wing imaginal discs compared to *pcm*^14^ (compare C with B) (n=13).

(D) Inhibiting apoptosis partially rescues the size of *pcm*^14^ wing imaginal discs. Reducing the copy number of *reaper*, *hid* and *grim* from 2 to 1 using the Df(3L)H99 deletion as a heterozygote (*pcm*^14^;*Df(3L)H99/+*) rescues the wing disc size from 45% to 81% that of wild-type (n≥31). Use of the Ark^82^ allele as a homozygote rescues wing disc size from 45% to 85% that of wild-type, showing that the adaptor protein Ark is required for much of the *pcm*^14^ induced apoptosis (n≥19). Over-expression of the Inhibitor of Apoptosis Protein DIAP1, using the 69B-GAL4 driver (*pcm*^14^;*GAL80ts/+;69B-GAL4/UAS-DIAP1) rescues the size of *pcm*^14^ discs from 45% to 67% that of wild-type. (n≥19). (** = p<0.0001). Inhibiting apoptosis in the *pcm*^WT^ background increases wing disc size by 5% using the Df(3L)H99 deletion (p<0.05), 12% using the Ark^82^ homozygous mutation (p<0.0001) and 15% using the UAS-DIAP1 (p<0.0001). Error bars represent 95% confidence limits.
To further corroborate that the ectopic apoptosis in pcm\textsuperscript{14} wing imaginal discs is occurring through the pathway in Figure 5.1, the inhibitor of apoptosis protein DIAP1 was overexpressed throughout the disc during larval development. This was achieved using the \textit{UAS-DIAP1} construct and the \textit{69B-GAL4} driver. To prevent DIAP1 ectopic expression during embryogenesis, GAL80\textsuperscript{ts} was also used (Figure 5.2C). pcm\textsuperscript{14}; GAL80\textsuperscript{ts}/+; 69B-GAL4/UAS-DIAP1 larvae were moved from 19°C to 29°C 48h after egg lay so that the \textit{UAS-DIAP1} would be turned on during the L1 larval period. This rescued wing imaginal disc size to 67\% that of wild-type (Figure 5.3D), which is a 40\% rescue in size ((67-45)/(100-45)*100). Wing imaginal disc size of pcm\textsuperscript{WT}; GAL80\textsuperscript{ts}/+; 69B-GAL4/UAS-DIAP1 L3 larvae increased by 15\% compared to pcm\textsuperscript{WT}, under the same conditions (Figure 5.3D).

The above experiments provide evidence that the increased apoptosis in pcm\textsuperscript{14} wing imaginal discs is directly responsible for the reduced growth of the wing imaginal discs and is indeed being induced through the pathway depicted in Figure 5.1.

\textbf{5.4 The delayed development of pcm\textsuperscript{14} wing imaginal discs is rescued by inhibiting apoptosis}

In order to test whether the apoptosis was also the cause of the delayed development of the pcm\textsuperscript{14} discs, pcm\textsuperscript{14}; \textit{Df(3L)H99/+} wing imaginal discs were stained with Wingless as in Figure 3.15. pcm\textsuperscript{14}; \textit{Df(3L)H99/+} wing discs at 120 hours AEL possess the characteristic Wingless expression pattern (Figure 5.4C) as seen in wild-type discs (Figure 5.4A). Therefore the delay in development of pcm\textsuperscript{14} discs is also rescued by inhibiting apoptosis. This demonstrates that it is apoptosis which is the cause of the delayed development of the discs and not \textit{vice versa}.
Figure 5.4. Inhibiting apoptosis rescues the delay in pcm14 wing imaginal disc development.

Inhibiting apoptosis in pcm14 larvae using the Df(3L)H99 as a heterozygote rescues the delay in wing imaginal disc development. This is shown by staining pcm14; Df(3L)H99/+ wing imaginal discs at 120 hours with the Wingless antibody (compare C with B) (n=13). Scale bar represents 100µm.
5.5 Inhibiting apoptosis fails to rescue pcm^{14} pupal lethality

In order to see if the inhibition of apoptosis in pcm^{14} developing larvae was able to rescue the lethality of pcm^{14} mutants, the phenotypes of the offspring from the crosses set up in Figure 5.2 were observed. As can be seen from Figure 5.5, no y^{1} pcm^{14}/Y;Df(3L)H99/+, pcm^{14}/Y;Ark^{82}/CyO-GFP or y^{1} pcm^{14}; GAL80^{ts}/+; 69B-GAL4/UAS-DIAP1 males were observed in the progeny. No y^{1} pcm^{14}/Y; Ark^{82} males could be observed as the Ark^{82} mutation is pupal lethal.

It therefore seems likely that other essential roles of Pacman exist in the developing larvae, in addition to regulating apoptosis. These roles could indeed be as equally important as regulating apoptosis, except that the disruption of these functions does not result in such an obvious phenotype in pcm^{14} mutants.
Figure 5.5. Inhibiting apoptosis in pcm14 developing larvae does not rescue pupal lethality.
The genotypes of the males (in which apoptosis is rescued) in the progeny of the crosses set up are shown. (A) When crossing the \textit{Df(3L)H99} deletion to pcm14, no round eyed, yellow bodied males are observed in the progeny. Therefore inhibiting apoptosis is not able to rescue the pcm14 lethality. (B) When crossing the \textit{Ark82} mutation to \textit{pcm14}, no round eyed, yellow bodied males were present in the offspring. All flies had curly wings, demonstrating that the \textit{Ark82} mutation causes pupal lethality when homozygous. (C) When inhibiting apoptosis with DIAP1 overexpression, no round eyed, yellow bodied males were present, demonstrating that this was not able to rescue \textit{pcm14} lethality. Bar eyed, yellow bodied males and round eyed males were present, indicating that the DIAP1 overexpression was not lethal under these conditions.
5.6 Is Pacman specifically degrading *reaper, hid* or *grim* mRNA?

The genetic evidence presented above indicates that the *pcm^{14}* mutation is causing apoptosis through the activation of the pro-apoptotic genes *reaper, hid* and *grim*. As Pacman is an exoribonuclease, one could hypothesize that Pacman could specifically target *reaper, hid* and *grim* mRNA with the consequence that the loss of Pacman would result in increased levels of these mRNAs and the subsequent induction of apoptosis. If this were true, then it would be expected that these mRNAs would increase at the post-transcriptional level in *pcm^{14}* mutants. Whereas the alternative hypothesis that Pacman is indirectly regulating apoptosis would most likely lead to a transcriptional increase in these mRNAs.

To test this, quantitative TaqMan q-RT-PCR was carried out to measure the levels of both the mature and pre-mRNA for *reaper, hid*, and *grim* in *pcm^{14}* and wild-type wing imaginal disc cells. Levels of pre-mRNA will be expected to increase in *pcm^{14}* cells if these genes are transcriptionally up-regulated. Therefore, if levels of mature mRNA are increased but levels of pre-mRNA are unaffected then this would suggest that these genes are post-transcriptionally regulated. Yet if any increase in mature mRNA is also seen at the level of pre-mRNA then this would suggest that these genes are transcriptionally regulated. Levels of mature mRNA were detected using pre-designed primer/probe assays. In order to detect levels of pre-mRNA, primers were designed across exon/intron boundaries or across the polyadenylation cleavage site for genes which do not possess introns (Figure 5.6).
Figure 5.6. Diagrammatic representation of the custom TaqMan primer/probe sets used to measure the pre-mRNAs of (A) hid and (B) reaper and (C) grim. Dashed box represents the 100nt sequence submitted to Life Technologies’ web-based Custom TaqMan Assay Design Tool. Actual sequences of primer/probes are listed.
5.6.1 *reaper* is up-regulated post-transcriptionally in *pcm*\(^{14}\) wing imaginal disc cells

As can be seen from Figure 5.7A, *reaper* mRNA is increased by 7.8-fold in *pcm*\(^{14}\) wing imaginal discs compared to wild-type. Interestingly levels of *pre-reaper* were not found to be significantly different in *pcm*\(^{14}\) cells compared to wild-type (p=0.0808). This therefore strongly suggests that *reaper* is a direct target of Pacman as *reaper* mRNA is post-transcriptionally up-regulated in *pcm*\(^{14}\) wing imaginal disc cells. As a control, levels of *reaper* and *pre-reaper* were also measured in *pcm*\(^{14}\); Df(3L)H99/+ wing imaginal discs. As these cells only have one copy of *reaper*, *hid* and *grim* one would expect these levels to be halved compared to *pcm*\(^{14}\) wing imaginal discs. Indeed, levels of mature *reaper* were reduced to 3.4 fold that of wild-type in *pcm*\(^{14}\); Df(3L)H99/+ wing imaginal disc cells (Figure 5.7A), which is a reduction of 56% (((7.8-3.4)/7.8)*100) compared with *pcm*\(^{14}\). This fits in with the genetic data that *pcm*\(^{14}\); Df(3L)H99/+ wing imaginal disc size is rescued by 65% (Figure 5.3D). However, unexpectedly levels of *pre-reaper* were not significantly reduced in *pcm*\(^{14}\); Df(3L)H99/+ compared to *pcm*\(^{14}\) (Figure 5.7A). This could possibly mean that the primer/probe assay designed specifically to detect *pre-reaper* is not specific. Due to the importance of this result in determining whether Pacman was directly regulating apoptosis by specifically degrading *reaper* mRNA, this needed to be corroborated independently.

In order to independently verify whether *reaper* was transcriptionally up-regulated in *pcm*\(^{14}\) wing imaginal discs, transcription from the *reaper* locus was measured using a previously characterised *reaper-GAL4* insertion (Miguel-Aliaga & Thor, 2004). If *reaper* was transcriptionally up-regulated then levels of GAL4 would be expected to increase in *pcm*\(^{14}\); *reaper-GAL4/+* wing imaginal discs compared with *pcm*\(^{WT}\); *reaper-GAL4/+*. Results in Figure 5.7B show that levels of GAL4 are significantly higher in *pcm*\(^{14}\); *reaper-GAL4/+* wing imaginal discs (3.5 fold) compared to *pcm*\(^{WT}\); *reaper-GAL4/+* (Figure 5.7B). Furthermore, levels of *pre-reaper* were increased by 1.8 fold in these discs (Figure 5.7B) and this increase was not shown to be significant compared to the increase in GAL4 (p=0.0533). This therefore indicates that *reaper* is transcriptionally up-regulated in *pcm*\(^{14}\) wing imaginal discs. However, this up-regulation does not account for the up-regulation seen at the level of mature mRNA as *reaper* was shown to increase by 8.6 fold in *pcm*\(^{14}\); *reaper-GAL4/+* wing imaginal discs compared to *pcm*\(^{WT}\); *reaper-GAL4/+* (Figure 5.7B).
Figure 5.7. *reaper* is up-regulated in *pcm*14 wing imaginal disc cells mainly at the post-transcriptional level.

(A) Levels of *reaper* mRNA increase 7.8-fold in *pcm*14 mutant wing imaginal discs compared with wild-type whereas levels of pre-*reaper* RNA are not significantly different. The increase in mature *reaper* RNA is halved to 3.4-fold in *pcm*14;*Df(3L)H99*/+ mutant wing imaginal discs, where there is only one copy of the *reaper*, *hid* and *grim* genes. (n=6 for wild-type and *pcm*14 and n=5 for *pcm*14;*Df(3L)H99*. *** = p<0.001. Error bars represent standard error). (B) Use of *reaper*-GAL4 (rpr-GAL4/+) as a transcriptional reporter shows that the *pcm*14 mutation results in an increase of 3.5 fold in levels of GAL4 mRNA in *pcm*14 wing imaginal discs (pcm14; rpr-GAL4/+) compared to wild-type (pcmWT; rpr-GAL4/+). This increase is not significantly different from the increase of 1.8 fold in pre-*reaper* RNA. Levels of mature *reaper* mRNA are substantially and significantly increased by 8.6-fold in *pcm*14; rpr-GAL4/+ wing imaginal discs compared to those from *pcm*WT; rpr-GAL4/+ discs. (n=4. *** = p<0.001 and * = p<0.05. Error bars represent standard error). (C) Levels of pre-*reaper* are reduced by roughly 2 fold in *pcm*WT; reaper-GAL4/+ and *pcm*14; reaper-GAL4/+ wing imaginal disc cells compared with *pcm*WT and *pcm*14 respectively. Since the reaper-GAL4/+ discs have one copy of the reaper gene compared to 2 in wild-type, this is to be expected. In *pcm*WT; reaper-GAL4/+ disc cells, pre-*reaper* RNA is reduced by 2.3-fold compared to the *pcm*WT control. In *pcm*14; reaper-GAL4/+ wing imaginal disc cells the levels of pre-*reaper* RNA are reduced by 2.1-fold compared to *pcm*14. (n=4 for *pcm*WT; reaper-GAL4/+ and *pcm*14; reaper-GAL4/+ and n=6 for *pcm*WT and *pcm*14. * = p<0.05. Error bars represent standard error.)
As a control to test for the specificity of the primer/probe assay for pre-reaper, levels of pre-reaper were measured in pcm14 and pcmWT with and without the reaper-GAL4 insertion. This insertion is 22bp up-stream of the reaper transcription start site and so should abolish transcription from this reaper locus. Therefore, levels of pre-reaper would be expected to be halved in pcmWT;; reaper-GAL4/+ and pcm14;; reaper-GAL4/+ compared to pcmWT and pcm14, respectively. Figure 5.7C shows that levels of pre-reaper were reduced 2.3 fold in pcmWT;; reaper-GAL4/+ disc cells compared to pcmWT and 2.1 fold in pcm14;; reaper-GAL4/+ discs compared to pcm14. This therefore confirms the reaper-GAL4 stock is as expected and that the pre-reaper primer/probe assay designed is specific for pre-reaper.

These results suggest that reaper is largely regulated by Pacman at the post-transcriptional level but also at the transcriptional level. It is possible that the transcriptional up-regulation of reaper is contingent upon an initial post-transcriptional increase in reaper in that ectopic expression of either reaper or hid can induce a transcriptional feed-back loop, causing a further increase in reaper expression at the transcriptional level (Shlevkov & Morata, 2012). In agreement with this is the observation that, for each biological replicate, those that showed the greatest increase in mature reaper RNA showed the greatest increase in the levels of GAL4.
5.6.2 *hid* is post-transcriptionally up-regulated in *pcm14* wing imaginal disc cells

Figure 5.8 shows that levels of *hid* mRNA are increased by 2-fold in *pcm14* wing imaginal discs compared to wild-type. Interestingly, levels of *pre-hid* were not significantly different (p=0.3634). This therefore indicates that *hid* is also post-transcriptionally regulated in *pcm14* wing imaginal disc cells and so is also a likely target for Pacman. Levels of *hid* were also reduced by roughly two fold in *pcm14*; *Df(3L)H99/+* wing imaginal discs compared with *pcm14*, although this difference only showed statistical significance if no correction for multiple comparisons was performed (i.e. if a t-test was used to compare *hid* levels in *pcm14* and *pcm14*;*Df(3L)H99, rather than an ANOVA). However, levels of *pre-hid* were not significantly reduced in *pcm14*; *Df(3L)H99/+* cells compared to *pcm14*. Unfortunately there is no *hid-GAL4* insertion to independently verify this result.

5.6.3 *grim* was not significantly up-regulated in *pcm14* wing imaginal disc cells

The levels of *grim* mRNA were very low and variable and showed no significant difference with those of wild-type (Figure 5.9). Levels of *pre-grim* were not reliably detected suggesting that *grim* is not actively transcribed in the wing imaginal discs.
Figure 5.8. *hid* is post-transcriptionally up-regulated in *pcm*14 wing imaginal disc cells compared to wild-type.

Levels of *hid* mRNA increase 2-fold in *pcm*14 mutant wing imaginal discs compared with wild-type, whereas levels of pre-*hid* RNA do not differ significantly. The levels of mature *hid* are reduced in *pcm*14;;*Df(3L)H99/+ wing imaginal discs, although this difference only showed statistical significance if no correction for multiple comparisons was performed (i.e. if a t-test was used to compare *hid* levels in *pcm*14 and *pcm*14;;*Df(3L)H99, rather than an ANOVA). (n=6 for wild-type and *pcm*14 and n=5 for *pcm*14;;*Df(3L)H99. *** = p<0.001 and * = p<0.05. Error bars represent standard error).

Figure 5.9. *grim* is lowly expressed and not significantly up-regulated in *pcm*14 wing imaginal disc cells compared to wild-type.

Levels of *grim* mRNA are not significantly different between wild-type and *pcm*14 wing imaginal discs. *grim* pre-mRNA could not be reliably detected. (n=6 for wild-type and *pcm*14 and n=5 for *pcm*14;;*Df(3L)H99. * = p<0.05. Error bars represent standard error).
5.7 Chapter summary

The main conclusions from this chapter are that the reduced growth and delayed development of the wing imaginal discs in pcm14 mutants are a direct consequence of an increase in apoptosis in these discs. This apoptosis was shown to occur through the intrinsic pathway depicted in Figure 5.1, which is initiated through an up-regulation of both reaper and hid mRNAs. These mRNA were shown to be mainly regulated at the post-transcriptional level, suggesting that these could be direct targets of Pacman. These observations are consistent with the hypothesis that Pacman is required to degrade reaper and hid mRNAs in wing imaginal disc cells in order to prevent apoptosis.

5.8 Chapter discussion

5.8.1 Is Reaper and Hid protein increased in pcm14 wing imaginal discs?

An increase of reaper and hid mRNA in pcm14 mutants would normally mean that Reaper and Hid proteins are also increased. However, this was not possible to test directly using Western blotting as the antibodies available gave only non-specific bands or no bands at all. Nevertheless, the results are completely consistent with the assumption that the increase in reaper and hid transcripts result in an increase in Reaper and Hid protein and that this protein is fully functional. For example, in pcm14 discs, where reaper is increased by 7.8-fold (Figure 5.7) and hid is increased by 2-fold (Figure 5.8) the size of the discs is 45% that of wild-type (Figure 5.3). Yet in pcm14 ;Df(3L)H99/+ discs, reaper is increased only 3.4-fold (Figure 5.7) and hid 1.6-fold (Figure 5.8), and these discs have been rescued in size by 67% (Figure 5.3). This strongly suggests that an increase in reaper and hid mRNAs results in an increase in Reaper and Hid protein. Similarly, our genetic data show that inhibiting the apoptosis pathway downstream of Reaper and Hid, by knocking out Ark and overexpressing DIAP1, rescues the size of pcm14 wing imaginal discs (Figure 5.3). This strongly suggests that Reaper and Hid are increased in pcm14 wing imaginal discs at the level of the protein, which by extension demonstrates that the increased reaper and hid transcripts are most likely translated.

If the increased reaper and hid transcripts are indeed translated, then usually this would imply that these transcripts are both capped and polyadenylated. Pacman/Xrn1 has been clearly shown to be a de-capping activator in that it has been shown to bind the decapping factor Dcp1 (Braun et al, 2012). Dcp1 associates with the decapping enzyme Dcp2,
therefore coupling decapping to 5'-3' degradation. In S2 cells, knockdown of pacman was shown to inhibit decapping, leading to an accumulation of capped transcripts (Braun et al, 2012). In pcm14 cells where no Pacman is present, decapping would therefore be expected to be impaired, which is consistent with the data suggesting that there is an increase in the level of Reaper and Hid protein.

Further evidence to support the argument that the up-regulated reaper transcripts are polyadenylated comes from the RNA-Seq experiment carried out by Dr. Chris Jones. The RNA for this experiment underwent poly(A) selection in order to remove ribosomal RNA. The results from this experiment showed that reaper was up-regulated by 4.5 fold in pcm14 wing imaginal disc cells compared to wild-type. Therefore, roughly half of the increased reaper transcripts are indeed polyadenylated, which strongly implies that these transcripts are translated.

Surprisingly, the RNA-Seq experiment showed that levels of hid mRNA went down in pcm14 wing imaginal disc cells compared to wild-type. The difference between this result and that obtained from the q-RT-PCR experiment above (Figure 5.8) is most likely explained by the fact that the RNA for the RNA-Seq was poly(A) selected. This therefore suggests that the increase in hid mRNA in pcm14 wing imaginal disc cells is not polyadenylated and is therefore less likely to be efficiently translated.

Another possible explanation however is that reaper and hid mRNAs do not need to be capped in order to be translated, as it has been shown that they possess Internal Ribosome Entry Sites (IRES) within their 5' UTRs (Hernandez et al, 2004; Vazquez-Piantzola et al, 2007). This is very interesting and will be discussed further in the discussion.

### 5.8.2 Are any additional apoptotic genes mis-expressed in pcm14 mutant wing imaginal disc cells?

To take full advantage of the RNA-Seq data obtained by Dr. Chris Jones on pcm14 and pcmWT wing imaginal discs, I specifically searched for components of the apoptosis pathway in the list of up and down regulated transcripts. None of the components down-stream of the pro-apoptotic RHG genes showed any mis-expression in pcm14 wing imaginal disc cells compared to wild-type. Interestingly, sickle, although lowly expressed was shown to be up-regulated by 10-fold in pcm14 cells. As this RNA is poly(A) selected this increase may well be contributing to the increased apoptosis in pcm14 mutants. It would therefore be interesting to determine whether this increase was transcriptional or post-transcriptional. Levels of the
two additional RHG genes Jafrac2 and HtrA2 were not mis-expressed in these cells. In agreement with the q-RT-PCR experiment above (Figure 5.9), levels of grim were expressed at very low levels in these cells.
6 Is reaper a direct target of Pacman?

The evidence presented so far supports the notion that ectopic apoptosis in pcm\textsuperscript{14} wing imaginal discs is caused by an up-regulation of the pro-apoptotic mRNA reaper and possibly also hid. Furthermore, as this up-regulation occurs primarily at the post-transcriptional level, a likely hypothesis is that reaper and hid mRNA are direct targets of Pacman and that Pacman is required in the wing imaginal disc cells to degrade reaper and hid mRNA to prevent apoptosis. However, an alternative hypothesis could be that the interaction between Pacman and reaper and hid is in-direct and that Pacman is regulating the expression of another gene, such as a miRNA or RNA binding protein for example, which itself is then regulating the expression of reaper and hid post-transcriptionally.

The most sophisticated and unbiased approach to show which mRNAs are being degraded by Pacman, would be to perform a Cross-Linking Immunoprecipitation-Sequencing (CLIP-Seq) experiment, to identify which mRNAs physically interact with Pacman. This involves cross linking Pacman to RNAs using U.V. irradiation, and then pulling down these complexes, using an antibody for Pacman. The RNAs are then extracted and RNA Sequencing is performed. However, due to time constraints it was not possible for me to carry out this experiment within the remaining duration of my PhD. This experiment is currently be carried out in the lab by Dr. Chris Jones. If reaper and hid are shown to be bound by Pacman then this would be direct evidence for the role of Pacman in the degradation of these pro-apoptotic mRNAs.

Another approach which could be taken would be to focus on the degradation of reaper and hid mRNAs. A common technique to test whether an exoribonuclease is directly degrading certain mRNAs is to measure the half lives of the mRNAs in wild-type and either mutant or knockdown cells. This can be achieved by inhibiting transcription within the cells and measuring the concentration of the mRNAs at certain time points following this. Although this type of experiment can be carried out using tissue culture cells (Braun et al, 2012), it has not (to my knowledge) been successfully carried out in whole tissues. Nevertheless, in view of the benefits this experiment would bring, this experiment was attempted. However, preliminary data indicated that the efficiency of the transcriptional inhibition was greater in pcm\textsuperscript{14} discs than wild-type. This was analysed by measuring levels of pre-hid and pre-reaper, which if transcription was shut off, should theoretically be reduced to zero. Although the levels of these pre-mRNAs did decrease in response to α-amanitin treatment, the pcm\textsuperscript{14} discs were affected more by the α-amanitin. This could be
due to the reduced size of these wing imaginal discs resulting in an increased surface area to volume ratio, thus facilitating entry of the α-amanitin to the pcm14 cells more efficiently. It was therefore decided that this experiment was not feasible using different sized wing imaginal discs.

Although this experiment could be carried out in cultured cells derived from wing imaginal discs, it is unlikely these cells truly reflect the situation of developing wing imaginal disc cells which are growing and communicating to each other. Therefore, it may not be the case that upon knockdown of pacman within these cells, reaper and hid would be up-regulated.

The technique I felt would be most appropriate to assess whether Pacman is required for the degradation of reaper and hid is named circularised Rapid Amplification of cDNA Ends (cRACE). This technique involves sequencing both the 5′ and 3′ ends of individual mRNAs and has been used to show that deadenylation precedes decapping in mammalian cells (Couttet et al, 1997), that histone mRNAs require oligouridylation for degradation (Mullen & Marzluff, 2008) and that uridylation can promote decapping, independent of deadenylation in S. pombe (Rissland & Norbury, 2009).

In addition to sequencing the ends of the mRNAs, this technique allows capped and decapped transcripts to be distinguishable from each other. It was decided that this technique would be used to sequence the 5′ and 3′ ends of the capped and decapped populations of reaper mRNA, in the wild-type and pcm14 wing imaginal disc cells. Due to time constraints hid mRNA was not assessed. This was because it is less likely that the increased levels of hid mRNA are causing the apoptosis as the RNA-Seq results suggest these transcripts do not possess a poly(A) tail (see 5.8.1). Using custom designed primer probes assays, it would also be possible to quantify the levels of reaper mRNA in the capped and decapped pools to determine whether the up-regulation of reaper mRNA is seen in the capped pool or the decapped pool. The aims of this experiment were therefore to address the following questions;

- Is reaper degraded only in the 5′-3′ direction?
- In pcm14 cells, does the 3′-5′ compensate for the lack of functional Pacman?
- Are the up-regulated reaper mRNAs capped?
6.1 Using circularised rapid amplification of cDNA ends (cRACE) to identify the 5' and 3' ends of reaper transcripts

The reaper gene is short and does not contain any introns. It is 901bp in length and the amino acid sequence is 65 amino acids long (therefore technically it would be considered a small Open Reading Frame (smORF)) and is only 7.7 kDa in size.

Total RNA was extracted from wild-type and pcm14 wing imaginal discs and each sample was then separated to create a capped and decapped pool. The capped pool was first incubated with Shrimp Alkaline Phosphatase (SAP), to remove the 5' phosphate from the decapped RNA, followed by Tobacco Acid Pyrophosphatase (TAP), to remove the 5' m7G cap from the capped RNA, leaving a 5' phosphate. In order to circularise the RNA the samples were then treated with T4 RNA ligase, which ligates the 5' phosphate with the 3' most nucleotide. The capped pool will therefore only contain circularised RNA which were capped within the original sample, whereas the decapped pool will only contain circularised RNA which were decapped. Gene specific primers were then used to reverse transcribe cDNA which is then amplified by two rounds of inverse PCR (20-25 cycles each) with nested primers to increase specificity. These transcripts are then cloned into E. coli so that individual transcripts can by sequenced (Figure 6.1). It is based on the assumption that each mRNA molecule has an equal chance of being sequenced and so the population of sequences represents the population of mRNAs from the extracted RNA.

Primers used in the reverse transcription and PCR steps were designed to be an equal distance from the 5' to 3' ligation so that there was no bias between 5'-3' and 3'-5' degradation products (Figure 6.2A). The size of the PCR product amplified by the F2/R2 primers, if the whole sequence is present, would be 850bp in length plus the length of the poly(A) tail (Figure 6.2B). The sequences of the primers used are shown in Figure 6.2C.
Total RNA is extracted and split into two pools. In order to create circularised capped RNA the capped pool is first treated with Shrimp Alkaline Phosphatase to remove the 5' phosphate from the decapped transcripts in this sample. The 5' m7G cap is then removed by Tobacco Acid Pyrophosphatase, leaving a 5' phosphate which acts as the substrate for the T4 RNA ligase during circularisation. In order to circularise the decapped RNA the decapped pool is simply treated with T4 RNA ligase as these transcripts already have a 5' phosphate for ligation. The circularised RNA is then converted into cDNA by reverse transcription and this is then amplified by PCR using nested primers to increase specificity. These individual sequences are then cloned into *E. coli* and sequenced.
Figure 6.2. Location of reaper primers used for the reverse transcription and PCR. (A) Circularised reaper transcript showing the location of the primers used in the reverse transcription and PCR steps of the cRACE. (B) Diagrammatic representation of the reaper PCR product amplified with the location of the primers shown. (C) Sequences of the primers used.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpr RT1</td>
<td>TTGTACTCTGGATTGTTGGAC</td>
</tr>
<tr>
<td>rpr F1</td>
<td>TGGACTATTGGTTTCCCCTGA</td>
</tr>
<tr>
<td>rpr F2</td>
<td>ATCCTCATTGCGATGGCTTG</td>
</tr>
<tr>
<td>rpr R1</td>
<td>AGCATGAGCCAAAACCAAAAC</td>
</tr>
<tr>
<td>rpr R2</td>
<td>ACAGTCACCACCTCAAGCC</td>
</tr>
</tbody>
</table>
In order to determine whether the steps leading up to the cloning and sequencing of the sequences had worked correctly, the products of the second round of PCR were run on an agarose gel (Figure 6.3). As the capped products are protected from 5'-3' degradation, one would expect these products to be full length if reaper was not able to be degraded 3'-5'. However, this was not the case as for both wild-type and pcm14 samples, a smear was seen from roughly 500-850bp. This suggests that these transcripts could be being degraded 3'-5', but as will be discussed later in this chapter, these products could also represent transcription products. For the wild-type decapped transcripts no band or smear is seen. This is not surprising as these transcripts will be rapidly degraded by Pacman, and 5'-3' degradation has been shown to be coupled to decapping (Braun et al, 2012). This suggests that more starting material is required in order to detect these transcripts but due to time restraints this was not possible. It was therefore decided that for the decapped pools, the two rounds of PCR would be repeated using 25 cycles rather than 20. For the pcm14 decapped transcripts a smear was observed, which suggests that there was more decapped reaper transcripts in the pcm14 wing imaginal disc cells than in wild-type. However, as the products are not full length and are all different sizes, this suggests that these could be being degraded 3'-5', although again the possibility of these being transcription products cannot be ruled out.
Figure 6.3. Amplified cRACE PCR products.
PCR products after 20 cycles of PCR using primers *rprF1/rprR1* followed by 20 cycles of PCR using primers *rprF2/rprR2*. A smear of roughly 500-850bp is observed for the capped transcripts and a larger smear for *pcm14* decapped transcripts. However, no band or smear is observed for *pcmWT* decapped transcripts.
6.2 *reaper* mRNA is transcribed from position -16

In order to determine the 5’ and 3’ ends of the sequences, they had to be mapped to the published sequence available on FlyBase. Firstly, the flanking sequence had to be removed, by mapping this to the sequence either side of the vector insertion site. In order to check both the orientation of the sequence and that the full length of the transcript had been sequenced, the F2 and R2 primer sequences were located to ensure they were at the ends of the trimmed sequence. Following this, the remainder of the *reaper* sequence was mapped, so that the 5’ and 3’ ends of the mRNA could be located and then the sequences were remodelled to represent the original linear sequence of that particular mRNA.

It was immediately observed that there were several differences between the published sequence on FlyBase and the actual sequence of mRNA present in the population of mRNAs sequenced. Any base changes which were only observed in individual sequences were ignored, as these were most likely either PCR or sequencing errors (or less likely transcription errors). However, there were certain alterations in the sequence which appeared in all sequences and so most likely represented the true sequence of *reaper* mRNA in these cells.

The most notable of these is that there was an extra 16 bases of sequence between the 5’ and 3’ ends in all transcripts that had their full 5’ ends present. This sequence could have originated from either the 5’ or 3’ end of the sequence, but it was noticed that this sequence was identical to the 16 nucleotide sequence upstream of the transcription start site. Therefore this result indicates that transcription actually starts at position -16 (Figure 6.4). This was observed in RNA extracted from both the *pcm14* and *pcmWT* wing imaginal discs and also OrR whole flies, indicating that this could be the genuine transcription start site and not an alternative transcription start site only observed in these wing imaginal disc cells.

In addition to this there were several single base substitutions, insertions or deletions and also an extra ACA trinucleotide repeat at position 199, which is directly upstream of the ATG start codon (Figure 6.4). However, none of these alterations affected the amino acid sequence of the Reaper protein.
Figure 6.4. Sequence alignment of the published reaper sequence taken from FlyBase and the actual sequence of reaper transcripts sequenced during the cRACE experiment.

reaper transcription starts at position -16 in the sequenced transcripts (bottom line) when compared to the published transcription start site taken from FlyBase (top line).

Several single base pair insertions, substitutions or deletions are shown. The substitutions which occur in the coding region (highlighted in red) do not alter the amino acid sequence of the protein.
6.3 Identifying capped and decapped transcripts

Once the sequences had been mapped and linear models of the original mRNA sequences created, it was observed that 18 out of 48 capped pcm\(^{14}\) sequences did not contain the full 5' ends of the sequence. This was very surprising for two reasons. Firstly, the 5' cap should protect the 5' end of the mRNA from any 5'-3' exoribonuclease activity and secondly there is no detectable cytoplasmic 5'-3' exoribonuclease activity in pcm\(^{14}\) cells (Figure 3.3).

The most likely explanation to explain the first part of this contradiction would be that these transcripts are in fact decapped and the conditions of the experiment have not been optimised correctly so that only capped transcripts are being circularised in the capped pool. In order to prevent circularisation of the decapped transcripts in the capped pool, the RNA is first treated with Shrimp Alkaline Phosphatase (SAP). This removes the 5' phosphate from the ends of the transcripts which have either been decapped or cleaved. The capped transcripts are protected from this enzyme by the 5' m\(^7\)G cap. This means that after treatment with Tobacco Acid Pyrophosphatase (TAP), which removes the 5' m\(^7\)G cap structure and leaves a 5' phosphate, only the transcripts which were initially capped from the extracted RNA will have the 5' phosphate required for ligation by T4 RNA Ligase.

When optimising the conditions of the experiment, RNA was extracted from whole OrR flies, as it is very time consuming to extract enough discs to obtain the desired amount of RNA for the experiment. As all capped transcripts from this pilot experiment contained their full 5' ends, it is likely that the 5' units of SAP incubated for 30min at 37\(^\circ\)C was sufficient to remove the 5' phosphate from all the decapped reaper transcripts in this population of RNA. This was also the case for the RNA extracted from pcm\(^{WT}\) wing imaginal discs. However, this does not seem to be the case for reaper RNA in pcm\(^{14}\) wing imaginal discs, which suggests that there is a dramatic increase in decapped mRNA in pcm\(^{14}\) cells. This is to be expected in that Pacman degrades mRNA after decapping.

Those transcripts that lacked the full 5' end sequence were presumed to be decapped transcripts for the reasons above. To check that this was a fair assumption, the 5' and 3' ends were compared between these transcripts and those that were sequenced from the pcm\(^{14}\) decapped pool. As can be seen from Figure 6.5A and B these sequences do appear similar, in that they are all missing both the 5' and 3' ends. These transcripts were therefore pooled together to produce the pcm\(^{14}\) population of decapped reaper transcripts.
Figure 6.5. *pcm*$_{14}$ transcripts from the capped pool which lack a complete 5' end are most likely decapped transcripts. Diagrammatic representation of the 5' most and 3' most base for each transcript sequenced for the *pcm*$_{14}$ decapped pool and the *pcm*$_{14}$ capped pool which lack a complete 5' end. Yellow box represents 5'UTR, red box the open reading frame and the blue box represents the 3'UTR.
6.4 Wild-type and pcm14 capped reaper transcripts lack the 3' end of the mRNA and do not have poly(A) tails

The 5' and 3' nucleotide at the ends of the wild-type and pcm14 capped transcripts were plotted on graphs and this can be seen in Figure 6.6A and B. All of these transcripts start at position -16 (as mentioned previously) except 3 wild-type transcripts that start at position -17, -18 and -19. From the 49 wild-type and 30 pcm14 transcripts sequenced, 40 wild-type and 25 pcm14 sequences were unique. However, only one wild-type and no pcm14 transcripts contain the full 3' end of the mRNA. The reason the 3' end of all these transcripts is downstream of position 516 is because the R2 primer is located at position 495-514 and so any transcripts with the 3' end up stream of this would not be amplified during the PCR. This suggests that these reaper transcripts are being degraded 3'-5' both in the wild-type and pcm14 cells. However, another explanation for this result is that these transcripts are in fact incomplete transcription products. As this RNA is total RNA it would contain RNA present in both the cytoplasm and the nucleus. As capping has been shown to occur co-transcriptionally (Moore & Proudfoot, 2009), there is no way to determine which of these transcripts are transcription products and which are 3'-5' degradation products.

In tissue culture cells it is possible to extract RNA purely from the cytoplasmic fraction. However this is much more difficult for wing imaginal discs and was not feasible in the time frame available. In order to select against transcription products during the PCR reaction, primers can be designed so that they overlap exon/exon boundaries, so that only spliced mature RNA would be amplified. However, as reaper does not contain introns, this was also not possible. It is therefore not possible to conclude anything with any certainty from these results.

It is interesting that these transcripts still contain their complete open reading frames (Figure 6.6), although as transcripts which contain 3' ends within the open reading frame will be lost this may just be bias. However, as the open reading frame is so short (195bp) compared with the 3'UTR (484bp), hypothetically this could suggest that 3'-5' degradation products could still be translated into functional Reaper protein if they are still associated with ribosomes. Although mRNA decay is thought to predominantly occur in P bodies, it has also been observed to occur co-translationally (Hu et al, 2009). Indeed, the observation that 5' mRNA targeted cleavage products could be uridylated, targeting them to the 5'-3' degradation machinery rather than simply being degraded 3'-5' lead to the hypothesis that 5'-3' decay rather than 3'-5' decay is required to prevent the translation of truncated
mRNAs (Shen & Goodman, 2004). It could therefore be that due to its short 5'UTR and open reading frame that reaper mRNA is required to be degraded 5'-3' in order to prevent unwanted translation.

It is worth noting that the one full length transcript, in the wild-type capped pool, contained 4 A nucleotides 3' of the poly(A) cleavage site. However, it is still not possible to determine at what stage of the RNAs life cycle this transcript was in as there are three different possibilities. The first is that this RNA is being adenylation in the nucleus following cleavage at the poly(A) site. The second is that this RNA is being deadenylated in the cytoplasm. The third is that this RNA is being transcribed past the poly(A) site prior to cleavage occurring. This possibility cannot be ruled out as the sequence in the genome following the reaper poly(A) cleavage site is AAAA.
Figure 6.6. Both wild-type and pcm<sup>14</sup> capped transcripts lack complete 3' ends. Diagrammatic representation of the 5' most and 3' most base for each wild-type and pcm<sup>14</sup> capped transcript sequenced. 40 from 49 of the wild-type transcripts and 25 from 30 of the pcm<sup>14</sup> transcripts were unique. Only one wild-type and no pcm<sup>14</sup> transcripts had a complete 3' end. Yellow box represents 5'UTR, red box the open reading frame and the blue box represents the 3'UTR.
6.5 Wild-type and pcm\textsuperscript{14} decapped reaper transcripts have incomplete 5' and 3' ends

The 5' and 3' nucleotides at the ends of the wild-type and pcm\textsuperscript{14} decapped transcripts were plotted on graphs and this can be seen in Figure 6.7A and B. The 5' and 3' ends of all the wild-type decapped transcripts mapped to the same nucleotide. This suggests that these sequences have all derived from the same mRNA transcript. This is likely to be the case as there were very little amounts of wild-type decapped transcripts in the RNA population (Figure 6.3).

The fact that there is very little decapped reaper transcripts in the population of RNA extracted from wild-type wing imaginal discs is not surprising, considering the presence of Pacman in these cells is likely to degrade these immediately once they have been decapped. The fact that this transcript is also missing its 3' end suggests that it is also being degraded 3'-5' in addition to 5'-3' which could be interesting. However this cannot be concluded with any certainty as only one transcript has been sequenced. In order to sequence a larger amount of decapped reaper transcripts in wild-type wing imaginal discs, a larger amount of RNA would have to be used.

It was surprising to observe that the pcm\textsuperscript{14} decapped transcripts were also missing both the 5' and 3' ends of the mRNA, as it has been shown previously (Figure 3.3) that there is no detectable cytoplasmic 5'-3' exoribonuclease activity in pcm\textsuperscript{14} cells. However, the most likely explanation for this finding is that these transcripts are being cleaved internally, most likely by the PIN domain of Dis3 (Lebreton et al, 2008; Schaeffer et al, 2009; Schneider et al, 2009). The alternative possibility is that these transcripts were in fact from the nucleus, and are being degraded by the nuclear 5'-3' exoribonuclease Xrn2/Rat1.

The fact that most of these transcripts are also lacking the coding region argues against the hypothesis stated earlier that reaper requires 5'-3' degradation to prevent unwanted translation of 3'-5' degradation products. However, due to the uncertainty of the localisation of these transcripts it is difficult to determine this with certainty. The reason that the 5' end is never downstream of position 382 and that the 3' end is never upstream of position 516 again resides from the position of the F2/R2 primers which would not amplify transcripts with 5' or 3' ends down or upstream of these positions respectively.
Figure 6.7. Both wild-type and pcm14 decapped transcripts lack complete 5' and 3' ends. Diagrammatic representation of the 5' most and 3' most base for each wild-type and pcm14 decapped transcript sequenced. All the 16 wild-type sequences are identical, suggesting that these were all amplified from the same individual mRNA transcript. From the 35 sequenced pcm14 transcripts, 27 were unique. Yellow box represents 5'UTR, red box the open reading frame and the blue box represents the 3'UTR.
One possible explanation for this result could be contamination with RNases. However, if this was the case the RNA would appear more fragmented due to the endonuclease activity in these enzymes and so the distribution of the sequences would be more spread out across the gene. Furthermore if this sample was contaminated with RNases then it would be expected that all the samples would be similarly affected since they were all treated the same and this was not the case. "RNase Zap" wipes were used to clean surfaces prior to each part of the experiment and only RNA grade solutions and tips were used. It is therefore unlikely that this is the reason for this surprising result. Also, from the shape of the transcripts, it does appear that these transcripts are being degraded in both directions as the distance of each transcript end from the end of its 5' end is roughly equal the end of its 3' end. This observation is supported by the scatter diagrams in Figure 6.8 which show that the mean 5' end is position 313 and the mean position of the 3' end is 594, which is 307 nucleotides away from the 3' end. However, if this were true, this would argue that the pcm14 decapped transcripts are being degraded 5'→3', rather than being cleaved internally by Dis3. This would therefore suggest that these transcripts have derived from the nucleus where they are being degraded by Xrn2. It is unlikely that Xrn2 is localised to the cytoplasm in pcm14 cells, or that there is an additional cytoplasmic 5'→3' exoribonuclease which has yet to be discovered, as no detectable 5'→3' cytoplasmic activity is observed in pcm14 larvae (Figure 3.3).

Due to the uncertainty of the localisation of these transcripts nothing can be concluded with any certainty from these results. It would be possible to perform this experiment in cultured wing imaginal disc cells in which pacman has been knocked down. These cells could then be fractionated into nuclear and cytoplasmic fractions so that the experiment could be carried out on RNA extracted from the cytoplasmic fraction only. Using tissue culture would also permit larger amounts of starting RNA to be used so that decapped reaper transcripts could be detected in wild-type cells.
Figure 6.8. Scatter plots showing transcript length, 5’ most end and 3’ most end of all the transcripts sequenced. Error bars represent standard deviation.
6.6 Chapter summary

Unfortunately, none of the aims of this chapter have been met. Due to technical reasons, no solid conclusions about the degradation of *reaper* in wild-type and *pcm*\(^{14}\) wing imaginal disc cells can be made. The observation that *reaper* transcription starts at position -16 is interesting, but does not address the hypothesis being tested. In order for this experiment to work it would be beneficial to carry this out in tissue culture cells rather than wing imaginal discs. This is for two reasons. Firstly it is important to be able to separate the cytoplasmic and nuclear fractions of RNA and this is far easier to do so in cultured cells. Also, due to the instability of *reaper*, large amounts of RNA would be needed so that this experiment can be optimised correctly and decapped *reaper* transcripts can be more accurately detected. Obviously, if this were to be carried out in tissue culture, the expression of *reaper* in wild-type and *pacman* knockdown cells would first need to be determined. Only once this experiment has been optimised can it be used to reliably quantify whether the up-regulated transcripts are capped or decapped by q-RT-PCR. However, the fact that decapped transcripts were identified in *pcm*\(^{14}\) cells, but not wild-type, suggest that at least some of the increased *reaper* transcripts in *pcm*\(^{14}\) wing imaginal disc cells are not capped.

6.7 Chapter discussion

6.7.1 Are *reaper* transcripts undergoing 3'-5' decay

The results presented above are consistent with the 3'-5' degradation pathway being involved in the decay of *reaper* and also that the endonuclease activity of Dis3 is involved. However, as the subcellular location of these transcripts cannot be determined, no solid conclusions can be made. For example, there is no determining whether the capped transcripts are either 3'-5' degradation intermediates or incomplete transcription products. Further work is therefore required to fully understand the nature of *reaper* degradation.

It would be interesting to take this into tissue culture cells in which the half life of *reaper* could also be investigated by inhibiting transcription in these cells. Experiments could also be carried out to investigate the nature of the up-regulated *reaper* transcripts (presuming *reaper* is up-regulated in these cells upon *pacman* knockdown), in regards to whether these transcripts are capped or polyadenylated. Polysome profiling could also be carried out to determine whether these transcripts are likely translated or not.
7 Discussion

7.1 Summary of main findings

7.1.1 Characterisation of pcm14 phenotypes

The focus of chapter 3 was to further characterise the phenotypes observed in pcm14 mutants. The results demonstrated that Pacman function was required both for the viability of the organism and also for the correct growth of the wing, leg, haltere and eye imaginal discs. Furthermore it was shown that this requirement was dependent on the exoribonuclease activity of Pacman and that knockdown of pacman using RNAi within the discs also caused smaller wings and wing venation phenotypes. The requirement of Pacman was shown to be specific for the imaginal discs, in that the growth of the larva was unaffected. However, the larval period was shown to be increased in pcm14 mutants, most likely as a result of the reduced growth and development of the imaginal discs causing a delay in the larva reaching critical size. Investigation into the temporal requirement of Pacman suggested that the critical period of development in which Pacman is required, for correct wing development, is late L3.

7.1.2 Reduced growth of the wing imaginal discs is caused by an increase in apoptosis

The experiments in Chapter 4 further investigate the reduced growth of the wing imaginal discs in pcm14 mutants. Mosaic analysis was used to demonstrate the wing imaginal disc cells possess a cell autonomous requirement for correct growth and that loss of Pacman within the disc cells caused an increase in apoptosis in the wing pouch region of the disc. This also resulted in compensatory proliferation within the discs, but clearly this was not sufficient to prevent the substantial loss of wing imaginal disc tissue. Chapter 5 went on to show that the increase in apoptosis was directly responsible for the reduced growth of the discs and that the apoptosis was occurring through the intrinsic pathway depicted in Figure 5.1.
7.1.3 Loss of Pacman results in a post-transcriptional up-regulation in reaper and hid mRNA

In order to investigate whether the regulation of apoptosis by Pacman was direct or indirect, the hypothesis that Pacman could specifically target pro-apoptotic mRNAs in the wing imaginal discs was tested. To this end levels of pre-mRNA and mature mRNA of the pro-apoptotic genes, reaper, hid and grim were measure by TaqMan q-RT-PCR. These experiments showed that both reaper and hid were post-transcriptionally up-regulated in pcm14 wing imaginal discs, although reaper was also slightly transcriptionally up-regulated. These results suggest that Pacman could be required to degrade these mRNAs in wild-type wing imaginal discs to prevent apoptosis occurring. cRACE was then used to determine whether reaper mRNA required the 5'-3' pathway for degradation or whether the 3'-5' pathway could compensate in the absence of Pacman. However, the results of these experiments were inconclusive as it was not possible to determine between incomplete transcription products and 3'-5' degradation products.
7.2 Is Pacman directly regulating apoptosis?

There are two hypotheses which could explain why there is extensive apoptosis in pcm14 wing imaginal disc cells. The first is that it is an indirect effect caused by the disruption of an important cellular process, such as cytoplasmic 5′-3´ mRNA degradation. This could indeed cause stress to the developing cells and defects in cellular processes. Indeed, mutants of Xrn1 is S. cerevisiae do indeed show pleiotropic phenotypes such as defects in chromosome stability and microtubule function (Jones et al, 2012b). It is possible that such defects could arise in pcm14 cells and this has not been directly investigated as it was decided that the simplest hypothesis to test first was whether Pacman was directly affecting apoptosis (see below). Such stress could indeed cause the cell to undergo apoptosis and this would most likely occur through the transcriptional upregulation of the pro-apoptotic genes reaper, hid and grim (Figure 7.1). In support of this hypothesis is that only the wing pouch cells are undergoing apoptosis in late L3 larvae and these cells are indeed most sensitive to apoptotic stimuli, such as ionising radiation (personal communication with Juan Pablo Couso).

However, if this were the case then the results from Figure 5.7 and 5.8 would be expected to show a transcriptional rather than a post-transcriptional up-regulation of reaper and hid. Although reaper is also transcriptionally up-regulated, the majority of this up-regulation is occurring at the post-transcriptional level and it could be that this post-transcriptional up-regulation is driving the transcriptional up-regulation through a feed-back loop as shown in (Shlevkov & Morata, 2012). A post-transcriptional up-regulation of reaper and hid would therefore support the model proposed in Figure 7.2 that Pacman is required to degraded reaper and hid mRNA in wing imaginal disc cells, in order to prevent apoptosis and therefore allow the correct growth and development of the discs to occur.

This would therefore support the hypothesis that Pacman is indeed directly regulating apoptosis, although it could be said that the role of Pacman in the regulation is more indirect and that the mechanism in which these mRNAs are directly targeted to the 5′-3´ decay pathway is the key to understanding the importance of this regulation. Despite this, if Pacman is directly required to degrade these pro-apoptotic mRNAs in the wing disc, then this work still provides novel support for the widely ignored layer of RNA stability in the regulation of apoptosis. However, more work is required to demonstrate whether reaper and hid are indeed regulated post-transcriptionally and if so to determine the mechanism in which Pacman is directly regulating the expression of these genes.
A) Wild-type

- reaper
- Transcription
- pre-mRNA
- RNA processing
- mature mRNA
- Nucleus
- Cytoplasm
- RNA export
- RNA storage
- Translation

Low levels of Reaper allows Cell survival

B) pcm^{14}

- Cell stress
- reaper
- Transcription
- pre-mRNA
- RNA processing
- mature mRNA
- Nucleus
- Cytoplasm
- RNA export
- RNA storage
- Translation

Increased levels of Reaper leads to apoptosis
Figure 7.1. Model of the hypothesis that Pacman is indirectly required in pcm14 wing imaginal disc cells to prevent apoptosis occurring.
(Figure on previous page)

(A) In wild-type cells, in the absence of apoptotic stimuli, reaper transcription is relatively low and mature mRNA is rapidly degraded to prevent high levels of Reaper protein being produced, preventing the induction of apoptosis. (B) In pcm14 cells however, the cell is under stress conditions and this leads to a transcriptional activation of reaper, which leads to increased levels of Reaper protein and the induction of apoptosis.

Figure 7.2. Model of the hypothesis that Pacman is directly required to degrade endogenous levels of reaper and hid in order to prevent apoptosis.
(Figure on following page)

(A) In wild-type cells, reaper and hid are targeted to the 5'→3' degradation pathway to ensure levels are not high enough to lead to the induction of apoptosis. (B) In pcm14 cells, the lack of functional Pacman means levels of these mRNAs will increase. Although these mRNAs may be predominantly decapped, they have been shown to be able to undergo cap independent translation, which will cause an increase in levels of Reaper and Hid protein and will ultimately lead to an increase in cell death.
A) Wild-type

- **reaper**
- pre-mRNA → Transcription → Nucleus → mature mRNA → RNA processing → Cytoplasm → RNA export

Translation

Targeted to the 5'-3' pathway

Dcp2

Pacman

Low levels of Reaper protein leads to cell survival

B) pcm

- **reaper**
- pre-mRNA → Transcription → Nucleus → mature mRNA → RNA processing → Cytoplasm → RNA export

Translation

Targeted to the 5'-3' pathway

Dcp2

Increased levels of Reaper protein causes apoptosis

[Diagram showing molecular processes related to Reaper protein in wild-type and pcm conditions]
7.3 Are reaper and hid direct targets of Pacman?

The fact the both reaper and hid are post-transcriptionally up-regulated in pcm\(^{14}\) wing imaginal discs supports the hypothesis that these mRNAs are direct targets of Pacman. However, there are still remaining questions that need to be addressed. Firstly, what are the mechanisms by which Pacman might be specifically affecting the levels of hid and reaper mRNA? Why are these particular mRNAs affected in pcm\(^{14}\) mutant cells and why are the effects of the mis-expression of these mRNAs seen more strongly than others.

The answers to these questions depends on the mechanism in which these mRNAs require Pacman for degradation. There are two possible explanations for this. The first is that Pacman is directly targeted to hid and reaper mRNA, either through miRNAs or RNA binding proteins, which means in the absence of Pacman, levels of these mRNAs increase. The second is more of a passive mechanism, in that reaper and hid mRNAs are required to be degraded by the 5'-3' pathway, rather than the 3'-5' pathway (or at least have a strong preference for this pathway), so that again, in the absence of Pacman levels of these mRNAs increase.

7.3.1 Are reaper and hid directly targeted to Pacman through cis elements within their 3'UTRs

The most common mechanism in which mRNAs can be specifically targeted to the degradation machinery is through either miRNAs or RNA binding proteins specifically binding to cis elements within the 3' UTRs of the mRNA (Jones et al, 2012b). The most common RNA binding proteins that perform this role are the AU rich element binding proteins (AREs). These have been shown to target mRNAs to either the 5'-3' or 3'-5' pathway or sometimes both (Chen et al, 2001; Lykke-Andersen & Wagner, 2005; Murray & Schoenberg, 2007; Stoecklin et al, 2006). miRNA mediated degradation of target mRNAs has been shown to require the decapping machinery (Behm-Ansmant et al, 2006; Eulalio et al, 2007c; Nishihara et al, 2013; Pressman et al, 2012; Rehwinkel et al, 2005a), indicating a requirement for 5'-3' degradation. Interestingly the 3'UTRs of reaper and hid do contain predicted and validated miRNA binding sites (Brennecke et al, 2003; Ge et al, 2012; Hilgers et al, 2010; Stark et al, 2003; Xu et al, 2004). These binding sites are shown in Figure 7.3 which has been taken from Target Scan. The 3'UTR of hid is much larger than reaper and has many more miRNA binding sites than reaper. reaper however only contains one binding site within its 3'UTR and this has been shown to be targeted by miRNAs within the miR-2 seed family. It has also been shown that Dcp1 is required for the repression of a reporter
construct containing the *hid* 3’UTR, in the *Drosophila* eye disc (Pressman et al, 2012), suggesting that mRNA decapping and subsequent 5’-3’ decay is required *in vivo* for repression of this mRNA by either miRNAs or RNA binding proteins.

In order to determine whether the regulation of *reaper* and *hid* by Pacman is mediated through the 3’UTRs of these mRNAs, 3’UTR reporter constructs could be used as in (Pressman et al, 2012) and (Stark et al, 2003). These reporter constructs consist of an open reading frame for GFP under the regulation of a specific 3’UTR. These could be expressed in wild-type and *pcm*¹⁴ wing imaginal disc cells. If Pacman is regulating *reaper* or *hid* through sites within their 3’UTRs, you would expect levels of GFP to be higher in the *pcm*¹⁴ discs. If this is shown to be the case, reporter constructs could be used with mutations in the binding sites for either predicted or validated miRNAs in order to determine the miRNAs involved. This should alleviate any up-regulation of GFP shown in the *pcm*¹⁴ discs.
Figure 7.3. Diagrammatic representation of the 3'UTRs for (A) reaper and (B) hid, showing predicted and validated miRNA binding sites taken from TargetScanFly release 6.2.
7.3.2 Do reaper and hid require degradation by the 5'-3' pathway or can the 3'-5' pathway compensate

In order to address this question cRACE analysis was carried out in chapter 6. However, the results of these experiments were inconclusive, in that it was not possible to determine between 3'-5' degradation products and incomplete transcription products. In order to overcome this, the experiment needs to be completed on cytoplasmic fractions rather than total RNA. This is difficult to achieve in wing imaginal discs therefore it may be necessary to carry out this experiment using tissue culture cells derived from wing imaginal discs.

7.4 Are the increased levels of reaper and hid translated?

Although these experiments have been unable to show directly that the increased levels of reaper and hid correlate with increased levels of Reaper and Hid protein, the genetic evidence presented in this thesis strongly suggests they are. Unfortunately there are no available antibodies which reliably detect either Reaper or Hid. However, the fact that reaper and hid mRNA levels are increased in pcm14 cells which are undergoing apoptosis, and that this apoptosis is alleviated by reducing the levels of reaper and hid and also by inhibiting the apoptosis pathway downstream of Reaper and Hid, strongly suggests that there is an up-regulation of Reaper and Hid protein also, as increased levels of reaper and hid mRNA alone would not cause an increase in apoptosis.

7.4.1 Are increased levels of reaper and hid polyadenylated?

For the majority of cellular mRNAs, translation is cap dependent and requires a poly(A) tail. A common criticism of this work is based on the position of Xrn1/Pacman within the 5'-3' degradation pathway (Figure 1.2A). As Pacman normally degrades mRNAs after deadenylation and decapping, preventing degradation of these mRNAs by mutating pacman would lead to an accumulation of decapped and deadenylated mRNAs. As these mRNAs will unlikely be translated it raises the question of how these increased mRNAs lead to the phenotypes seen in pacman mutants. On the other hand it can be argued that the phenotypic data observed in pacman mutants, support the notion that there must be some mechanism in which certain mRNAs can be preferentially up-regulated and translated in pacman mutants.

Interestingly the RNA-Seq recently carried out by Dr. Chris Jones on poly(A) selected RNA from wild-type and pcm14 wing imaginal discs shows that there is a 4.5 fold up-regulation of
polyadenylated reaper mRNA in pcm14 cells compared with wild-type. Therefore it appears that roughly half of the up-regulated reaper mRNA in pcm14 cells does indeed possess a poly(A) tail. hid however was actually shown to be down-regulated, suggesting that the up-regulated hid mRNA seen in the q-RT-PCR experiments did not possess a poly(A) tail. It therefore seems likely that the increased levels of reaper but not hid are translated into protein.

### 7.4.2 Pacman is able to act as a decapping factor

A recent finding from Elisa Izaurralde’s lab has shown that Pacman/Xrn1 is able to act as a decapping factor by binding to Dcp1 in Drosophila and Edc4 in human cells (Braun et al, 2012). In addition to the role of Pacman/Xrn1 to act as a 5'-3' exoribonuclease, (Braun et al, 2012) showed that Xrn1/Pacman was also a bona fide decapping activator in both Drosophila and human tissue culture cells. Both knocking down and overexpressing pacman in tissue culture cells caused an accumulation of capped transcripts, although these transcripts lacked a poly(A) tail. It seems that overexpression of Pacman was able to inhibit decapping by interfering with the stoichiometric relationship of the decapping machinery. Indeed it has been shown that Dcp1 acts as a trimer (Tritschler et al, 2009) and so overexpression of Pacman could lead to the disruption of this complex if it is binding in a 3:1 ratio. Interestingly they showed that inhibition of decapping was stronger by overexpressing Pacman than knocking it down, most likely due to redundancy between other decapping factors, and so it is not clear how much decapping will be inhibited in pcm14 and pcm5 cells which lack the Dcp1 binding domain. However, the fact that the phenotypes in pcm5 are more severe than pcm13, which is a similar size deletion but without deleting the Dcp1 binding motif, suggest it is important. This therefore suggests that decapping is likely to be impeded in pcm14 cells at least slightly and so some of the mRNAs that increase in response to the pcm14 mutation should be capped.

Although this would need to be shown directly to occur in vivo, the role of Xrn1/Pacman as a decapping factor in Drosophila is supported by the fact that overexpression of Pacman in wild-type cells, with the act-Gal4 driver, causes pupal lethality and this was shown to be independent on the exoribonuclease activity of Pacman as this also occurred when overexpressing the UAS-pcmNO construct (Figure 3.6C), which suggests Pacman has an additional function. Also, the results obtained in Figure 3.9D show that overexpressing a nuclease dead pacman construct (UAS-pcmNO) caused a dominant negative effect in pcm14 larvae. This can be explained by the model shown in Figure 7.4, which shows that in pcm14
cells, 5’-3’ cytoplasmic exoribonuclease activity is abolished and decapping is also slightly inhibited. This would lead to the accumulation of transcripts that are not able to be degraded, but presumably most of these transcripts are decapped and so are less likely to be translated. Overexpressing a nuclease dead Pacman in these cells will not rescue any cytoplasmic 5’-3’ degradation but could further impede decapping, which could lead to a further increase in decapped transcripts, which are more likely to be translated.
Figure 7.4. Hypothetical model explaining the dominant negative effect of nuclease dead Pacman overexpression by its role as a decapping factor. 

(A) In wild-type cells, Pacman is able to bind Dcp1 (which acts as a trimer), in order to stimulate decapping and couple this to 5’-3’ degradation. 

(B) In $pcm^{14}$ cells, 5’-3’ degradation is abolished and decapping activity may also be slightly inhibited, leading to the accumulation of predominantly decapped transcripts. 

(C) In $pcm^{14}$ cells in which the nuclease dead Pacman is being overexpressed, 5’-3’ degradation is still abolished, but decapping is likely to be further inhibited, which could lead to the accumulation of predominantly capped transcripts.
7.4.3 *reaper* and *hid* are able to undergo cap independent translation

An alternative and very interesting hypothesis is that the increased levels of *reaper* mRNA are being translated in a cap independent manner. It has been shown that *reaper* (Hernandez et al, 2004) and also *hid* and *grim* (Vazquez-Pianzola et al, 2007) mRNAs are still efficiently translated in cells lacking eIF4E activity and that reporters possessing the 5'UTRs of these genes were translated independent of the presence of a 5' m7G cap. The mechanism in which this is achieved is not clear, as the *reaper* 5'UTR is relatively short and lacks any obvious secondary structure so does not appear to be a classical IRES. Despite this, *reaper* mRNA was still associated with polysomes in cells undergoing apoptosis in which global cap dependent translation initiation is impeded (Hernandez et al, 2004). Therefore it is likely that the increased levels of polyadenylated *reaper* mRNA would be translated in these cells independent on whether they possessed a 5' m7G cap. This could therefore explain why the phenotypic effects of the *pcm14* mutation is more reliant on the up-regulation of *reaper* and possibly *hid*, compared to the roughly 450 mRNAs that were shown to be significantly up-regulated by more than 2 fold in *pcm14* wing imaginal discs (from the RNA-sequencing experiment carried out by Dr. Chris Jones).

In order to test whether these increased levels of *reaper* and *hid* are translated, polysome profiling could be carried out. This technique involves the separation of the RNA bound to multiple ribosomes (polysomes) which are regarded to be undergoing efficient translation and the RNA bound to either a single 40S, 60S or 80S ribosome (monosomes) which are not regarded as being actively translated. By comparing the levels of *reaper* and *hid* mRNAs within the polysome fractions between wild-type and *pcm14* cells would determine whether the increased levels of these mRNAs are being actively translated.

This observation could have implications for further work investigating the requirement of Pacman for *Drosophila* development. If indeed the phenotypes of *pcm14* mutants are more dependent on the ability of the increased mRNAs to be translated rather than which mRNAs are more abundant and by how much then perhaps an important experiment to carry out, to complement the RNA-Seq results, would be polysome profiling followed by RNA-Seq. This would therefore allow a genome wide analysis of which of the mRNAs increased in abundance are also translated.
7.5 Reaper is involved in global shutdown of translation

In addition to its role as an IAP antagonist, Reaper has also been shown to inhibit global translation (Holley et al., 2002; Yoo et al., 2002). Interestingly, this has been shown to occur through direct binding to the 40S ribosome (Colon-Ramos et al., 2006). It was shown that Reaper binding to the 40S ribosome reduced the ability of the 48S initiation complex to recognise the AUG start codon. Translation driven by the CrPV IRES, which requires no eIFs and does not undergo scanning was shown to be unaffected by this alteration (Colon-Ramos et al., 2006). However, it was not tested whether cellular mRNAs which possess IRESs, such as reaper itself, were affected. This would be particularly interesting if increased levels of Reaper protein were able to lead to preferential translation of reaper mRNA as this would mean only a small increase in reaper mRNA could commit the cell to apoptosis.
7.7 Developmental parameters of apoptosis

During the writing of this thesis an interesting paper was published investigating the sensitivity of cells to apoptosis during Drosophila development (Kang & Bashirullah, 2014). By expressing pulses of reaper under heat-shock control during different stages of development and then measuring survival rates they demonstrated that cells were only sensitive to increased reaper expression during embryogenesis and late L3-early pupal development. It was shown that 50 times higher doses of reaper were required to induce apoptosis in early L3 larvae compared with late L3. Interestingly they found that the apoptosis that was induced in cells during early L3 larvae was induced independent of apoptosome formation and that the increased sensitivity to apoptotic stimuli during late L3 larvae coincided with an increase in expression of both Dronc and Ark (Kang & Bashirullah, 2014).

This data supports several findings of this thesis, although also stimulates some additional questions. Firstly the fact that the sensitivity to apoptotic stimuli was greatly increased during the late L3 period fits with the temporal requirement of Pacman for wing development being late L3, as shown from the GAL80 experiment in Figure 3.17-19. This suggests that knockdown of pacman using RNAi is not sufficient to induce extensive apoptosis until the L3 stage of development and could therefore also explain why L3 wing imaginal discs in pcmRNAi larvae were not smaller than wild-type yet the adult wings were significantly reduced in size.

However, the fact that the wing imaginal discs in pcm14 L3 larvae are already significantly smaller by the late L3 stage and that the mosaic analysis experiment shows that wild-type cells have a growth advantage over pcm14 cells during early larval periods suggests that apoptosis is occurring in pcm14 mutants before late L3. This difference in results could be explained by the fact that Kang and Bashirullah induced pulses of reaper expression, whereas in pcm14 there is likely to be prolonged overexpression of reaper.

Furthermore the fact that Kang and Bashirullah found that the apoptosis which occurred prior to late L3 was shown to be independent on the formation of the apoptosome fits with the findings in Figure 5.3, that complete knockout of Ark only partially rescued the reduced size of pcm14 wing imaginal discs.
7.9 Implications for human disease

7.9.1 Is the regulation of RNA stability critical in preventing cancer?

Due to the high conservation of Xrn1 and also the apoptosis pathways, between humans and Drosophila, it would be interesting to determine whether Xrn1 plays a role in regulating the apoptosis pathway in human cells. If mRNA stability is directly regulating processes such as apoptosis and proliferation this could have direct implications for cancer. Indeed, there is evidence to support the idea that there is a network of RNA-protein interactions that regulates the stability of specific mRNAs, which contribute to cancer related cellular processes such as apoptosis and proliferation in human cells. Firstly, support for the role of mRNA stability in regulating apoptosis includes work showing that the deadenylases Ccr4a and Ccr4b can affect cell survival in MCF7 human breast cancer cells (Mittal et al, 2011). Furthermore, there is evidence that the overexpression of genes encoding proteins involved in the 5'-3' degradation pathway, such as hLsm1 and Dhh1 are associated with certain tumours such as pancreatic and colon (Mazzoni & Falcone, 2011). Furthermore, the 3'-5' exoribonuclease Dis3, has been identified as being frequently mutated in multiple myeloma by whole genome sequencing studies (Chapman et al, 2011) and mutations in Dis3 are also associated with Acute Myeloid Leukaemia (Ding et al, 2012) and the progression of melanoma (Rose et al, 2011). In addition, mutations in the gene encoding the exosome independent Dis3L2 were found associated with Perlman syndrome of overgrowth and susceptibility to Wilms tumour (Astuti et al, 2012). Most interesting and relevant to this study, is the observation that Xrn1 is expressed at low levels in several osteosarcoma cell lines, suggesting it could act as a tumour suppressor (Zhang et al, 2002). The Newbury lab has since confirmed these findings and intends to investigate this further.

The mechanism in which the mis-expression of genes involved in regulating RNA stability leads to the development of cancer is not clear. It is interesting that it appears the progression of cancer correlates with either mutations or low expression of exoribonucleases, whereas the findings in this thesis correlate low or absent expression of pacman with reduced growth. This was also found to be the case Dis3, in which the mutations associated with multiple myeloma were shown to retard growth when incorporated into both human (HEK293) cell lines and S. cerevisiae (Tomecki et al, 2013).
This suggests that these mutations are acting in a combinatorial role with other mutations involved in the development of cancer.

An interesting hypothesis based on these findings and the observation that *Xrn1* was expressed at low levels in osteosarcoma cell lines, is that low expression of *Xrn1* could be promoting compensatory proliferation in tumour cells which have lost the ability to undergo apoptosis. If indeed *Xrn1* is also regulating the apoptosis pathway in humans (either directly or indirectly), as it is in *Drosophila*, loss or knockdown of *Xrn1* in human cells could lead to the activation of the apoptosis pathway in these cells. If this occurred in cells which have already obtained mutations which prevent the activation of apoptosis but do not prevent compensatory proliferation (such as the effector caspases), then this could potentially lead to an increase in proliferation rather than reduced growth. This hypothesis is currently being investigated in the Newbury lab and is shown in Figure 7.3. The alternative explanation of these results, is that the low expression of *Xrn1* is actually a consequence of the increased proliferation in these cell lines. If *Xrn1* is required to degraded pro-apoptotic genes in human cells, then the cells might be down-regulating *Xrn1* activity in an to attempt to activate the cell death pathway to overcome the increased proliferation.

In addition, the importance of the post-transcriptional regulation of mRNAs imposed through the action of miRNAs is highlighted by the fact that both the dysregulation of miRNA expression and also the shortening of the length of 3'UTRs is frequently being linked with the development of cancer (Mayr & Bartel, 2009; Nicoloso et al, 2009; Zhang et al, 2007), so much so that they have become attractive candidates for biomarkers of cancer (Bartels & Tsongalis, 2009; Jones et al, 2012a). This is therefore further evidence that cytoplasmic mechanisms which regulate gene expression at the post-transcriptional level are of critical importance in preventing the development of cancer.
Figure 7.5. Diagrammatic representation of the hypothesis being tested in the Newbury lab explaining why *pacman* is expressed at low levels in several osteosarcoma cell lines. In *pcm*\textsuperscript{14} wing imaginal discs, there is an increase in apoptosis, which causes compensatory proliferation to occur, although not enough to compensate for the levels of apoptosis occurring. If either loss or knockdown of *Xrn1* in human cells also caused an increase in apoptosis, then this would also cause compensatory proliferation to occur. If this occurred in cells that also carried a mutation which would prevent apoptosis but not compensatory proliferation, such as in a gene encoding an effector caspase for example, this would lead to overgrowth and possibly tumour formation.
7.10 Future work

7.10.1 Measuring the half life of reaper

Although the half life of reaper was attempted to be measured by inhibiting transcription using alpha amanitin in the wing imaginal discs, another method which could be used would be to induce a pulse of reaper expression using the hs-rpr flies created in the Steller lab (White et al, 1996). These constructs have recently been shown to induce a pulse of reaper expression of roughly 100 fold, which returns to almost wild-type levels within 2h (Kang & Bashirullah, 2014). By crossing these constructs into pcm14 and wild-type flies and inducing a pulse of reaper expression in the L3 larvae, the pulse of reaper expression and the rate of decay could be measured by q-RT-PCR at several time points to determine the half life of reaper. If reaper is post-transcriptionally up-regulated in pcm14 wing imaginal discs it would be expected to take longer to return to wild-type levels in pcm14 cells compared to wild-type.

7.10.2 Using tissue culture to further investigate the regulation of reaper and hid by Pacman

The focus of this thesis was to investigate the role of the cytoplasmic 5'-3' mRNA degradation pathway for the development of the wing imaginal discs, so that the findings could have further implications for the development of multicellular organisms. However, as with every model organism/system, there are both advantages and disadvantages of using wing imaginal discs. The overall findings of this thesis have benefited from the developmental context in which the experiments were carried out, especially in that the phenotypic data strongly supports the biochemical experiments, which adds further strength to the findings. However, I feel that it would now be advantageous to further investigate the regulation of reaper and hid mRNA by Pacman within tissue culture cells derived from wing imaginal discs. The benefits of using tissue culture includes the ease of growing up significant amounts of tissue and also the ability to easily fractionate the cytoplasmic and nuclear fractions.
7.10.2.1 cRACE

Carrying out cRACE on cytoplasmic extracts from tissue culture cells would overcome the problem in distinguishing between 3'-5' degradation products and transcription products as the transcription products will be left within the nuclear fraction. Once this experiment is optimised it could address the following questions.

Does the degradation of reaper and hid occur only 5'-3' or is there redundancy with the 3'-5' pathway?

If reaper and hid were only degraded 5'-3' then both the capped and decapped transcripts in cells knocked down for pacman would be expected to be full length, whereas decapped transcripts in wild-type cells would not contain the full 5' end.

Are increased levels of reaper and hid capped?

In order to determine whether the increased levels of reaper and hid are capped, q-RT-PCR could be used to quantify whether the up-regulation of these mRNAs is seen in the capped or decapped pool (or both). In order to specifically measure levels of the capped or decapped transcripts, primer/probes could be designed that would only detect reaper or hid transcripts which have been circularised during the ligation reaction. If the increase in reaper and hid mRNAs were capped then one would expect an up-regulation of reaper and hid in the pcm14 capped pool compared to the wild-type capped pool, whereas if they were not capped the up-regulation would be expected to be seen in the decapped pool.

An alternative technique to determine whether the increased levels of reaper and hid are capped would be to treat the RNA with terminator nuclease, a 5'-3' exonuclease the degrades uncapped monophosphorylated RNAs, prior to carrying out the q-RT-PCR, as in (Braun et al, 2012). This would therefore degrade all uncapped mRNA and so if reaper and hid were still shown to be up-regulated this would suggest these transcripts are capped.

7.10.2.2 Polysome profiling

If the up-regulated reaper and hid transcripts were shown to be decapped this would suggest that these transcripts are either not translated or that they are translated in a cap independent mechanism, which has been shown previously (Hernandez et al, 2004; Vazquez-Pianzola et al, 2007). In order to investigate this polysome profiling could be carried out, followed by either q-RT-PCR or RNA-Seq. Using RNA-Seq would allow a genome wide view of which mRNAs which increase in pcm14 cells are also translated.
7.10.2.3 Using 3'UTR reporters to determine whether Pacman regulates reaper and hid through their 3'UTRs

In order to determine whether Pacman regulates reaper and hid through their 3'UTRs, reporter constructs containing the 3'UTRs of reaper and hid could be used in cells wild-type and in which pacman had been knocked down. If reporter activity is increased in pacman knockdown cells, this would demonstrate that Pacman is targeted to these genes through their 3'UTRs. In order to determine whether this is through direct miRNA binding, miRNA binding sites could be mutated in these reporter constructs to determine whether this alleviates the up-regulation seen in pacman knockdown cells.

7.10.3 Investigating the role of Pacman in additional Drosophila systems

Although this thesis has focused on the role of Pacman for the growth and development of the wing imaginal discs, there is reason to believe it is required for the development for several other systems in Drosophila. Firstly, the size of the leg, haltere and eye discs were also significantly smaller in pcm^14 L3 larvae compared to wild-type (Figure 3.13), suggesting that Pacman is required for the correct growth and development of all the imaginal discs. Interestingly, although pcm^14/Y; UAS-pcm^WT/+; 69B-GAL4/+ flies eclose as adults (Figure 3.6C and 3.7), the behaviour of these flies is abnormal and they die within a couple of days of eclosing. This suggests that Pacman could also be required for the correct development of the nervous system. It would also be interesting to investigate the role of Pacman during embryogenesis as it is known that pacman mRNA is maternally contributed (Till et al, 1998). This would require the creation of a maternal null mutant for pacman, which would likely have more severe phenotypes that pcm^14 mutants and could indeed be embryonic lethal.
7.11 Concluding remarks

The most interesting findings presented in this thesis suggest that Pacman directly regulates apoptosis through the specific degradation of the pro-apoptotic mRNAs reaper and hid. Although further work is needed to determine the mechanism in which Pacman is targeted to these mRNAs, this is the first time an exoribonuclease has been shown to directly regulate apoptosis. It would therefore be very interesting to determine whether the regulation of apoptosis by Xrn1/Pacman is also conserved in human cells as this would have implications for research into many diseases which are caused by the mis-regulation of apoptosis, such as cancer.
8 References


Anderson JS, Parker RP (1998) The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SKI2 DEVH box protein and 3' to 5' exonucleases of the exosome complex. *The EMBO Journal* **17**: 1497-1506


Bridges CB (1916) Non-disjunction as proof of the chromosome theory of heredity. *Genetics* 1: 1-52


Brogna S (1999) Nonsense mutations in the alcohol dehydrogenase gene of *Drosophila melanogaster* correlate with an abnormal 3’ end processing of the corresponding pre-mRNA. *Rna* **5**: 562-573


Chowdhury A, Mukhopadhyay J, Tharun S (2007) The decapping activator Lsm1p-7p-Pat1p complex has the intrinsic ability to distinguish between oligoadenylated and polyadenylated RNAs. *Rna* **13**: 998-1016

Chowdhury A, Tharun S (2009) Activation of decapping involves binding of the mRNA and facilitation of the post-binding steps by the Lsm1-7-Pat1 complex. *Rna* **15**: 1837-1848


Fraser AG, Evan GI (1997) Identification of a Drosophila melanogaster ICE/CED3-related protease, drICE. The EMBO Journal 16: 2805-2813

Fraser AG, McCarthy NJ, Evan GI (1997) drICE is an essential caspase required for apoptotic activity in Drosophila cells. The EMBO Journal 16: 6192-6199


He WH, Parker R (2001) The yeast cytoplasmic LsmI/Pat1p complex protects mRNA 3' termini from partial degradation. *Genetics* **158**: 1445-1455


Hsu CL, Stevens A (1993) Yeast cells lacking 5'-3' exoribonuclease 1 contain mRNA species that are poly(A) deficient and partially lack the 5' cap structure. *Molecular and Cellular Biology* **13**: 4826-4835


Johnson AW (1997) Rat1p and Xrn1p are functionally interchangeable exoribonucleases that are restricted to and required in the nucleus and cytoplasm, respectively. *Molecular and Cellular Biology* **17**: 6122-6130

Johnson AW, Kolodner RD (1995) Synthetic lethality of sep1 (xrn1) ski2 and sep1 (xrn1) ski3 mutants of *Saccharomyces cerevisiae* is independent of killer virus and suggests a general role for these genes in translation control. *Molecular and Cellular Biology* **15**: 2719-2727


Jones CI (2011) Post-transcriptional gene regulation by the exoribonuclease Pacman. Doctor of Philosophy Thesis, Clinical and Laboratory Investigation Division, Brighton and Sussex Medical School,


Larimer FW, Stevens A (1990) Disruption of the gene *XRN1*, coding for a 5'-3' exoribonuclease, restricts yeast cell growth. *Gene* **95**: 85-90


Lisi S, Mazzon I, White K (2000) Diverse domains of THREAD/DIAP1 are required to inhibit apoptosis induced by REAPER and HID in Drosophila. Genetics 154: 669-678


Muhlrad D, Decker CJ, Parker R (1994) Deadenylation of the unstable mRNA encoded by the yeast MFA2 gene leads to decapping followed by 5'→3' digestion of the transcript. Genes & Development 8: 855-866


Mullen TE, Marzluff WF (2008) Degradation of histone mRNA requires oligouridylation followed by decapping and simultaneous degradation of the mRNA both 5' → 3' and 3' → 5'. Genes & Development 22: 50-65


Nishihara T, Zekri L, Braun JE, Izaurralde E (2013) miRISC recruits decapping factors to miRNA targets to enhance their degradation. Nucleic Acids Research 41: 8692-8705


Orban TI, Izaurralde E (2005) Decay of mRNAs targeted by RISC requires XRN1, the Ski complex, and the exosome. Rna 11: 459-469

Ozgr S, Chekulaeva M, Stoecklin G (2010) Human Pat1b connects deadenylation with mRNA decapping and controls the assembly of processing bodies. Molecular and Cellular Biology 30: 4308-4323


Proudfoot NJ (2011) Ending the message: poly(A) signals then and now. Genes & Development 25: 1770-1782


Schwartz DC, Parker R (1999) Mutations in translation initiation factors lead to increased rates of deadenylation and decapping of mRNAs in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* 19: 5247-5256


Stevens A (1980) Purification and characterization of a *Saccharomyces cerevisiae* exoribonuclease which yields 5’-mononucleotides by a 5’ leads to 3’ mode of hydrolysis. *Journal of Biological Chemistry* **255**: 3080-3085


Tharun S (2009) Lsm1-7-Pat1 complex A link between 3' and 5'-ends in mRNA decay? RNA biology 6: 228-232


