Rho GTPases and Early Zebrafish Development

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PhD Thesis

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ABSTRACT

The work in this thesis examines the role of the Rho family of small GTPase proteins in vertebrate embryogenesis, using the zebrafish, *Brachydanio rerio*, as an experimental model. These proteins have been shown to mediate actin rearrangements underlying cell movement and morphology changes both *in vitro* and *in vivo*. Their function has been well-characterised in single cells, but less is known about their role in the movements of cells in whole sheets, such as those occurring in the processes of epiboly and gastrulation in embryogenesis.

Firstly, the cloning of two, previously unknown members of the zebrafish Rho GTPase family is described - a full length *RhoA* homologue and a partial fragment of *Racl*. The expression patterns of both of these genes is determined over the course of embryogenesis, at several stages from the 4 cell stage embryo to the 72 hour larva. Additionally, the expression patterns of these genes in the adult brain are determined.

To address the functioning of the Rho pathway *in vivo*, synthetic mRNA transcripts encoding differing forms of human RhoA or the Rho-blocking C3 exoenzyme were injected into 2-4 cell stage embryos. The resultant phenotypes are presented, as analysed by in situ hybridisation, immunohistology and examination of embryonic morphology. Injection of constitutively active V14Rho results in a defective epiboly phenotype which is resolved into cyclopia and which phenocopies the silberblick/Wnt11 mutant phenotype. Blocking Rho function with C3 has a dramatic and rapid effect on embryogenesis, affecting cellularisation and axial polarity in the early embryo and resulting ultimately in death.

The final chapter deals with the characterisation of a mutant strain of fish arising from an ENU mutagenesis screen. Point mutations chemically induced in male germlines were bred to homozygosity over a three generation scheme and the F3 progeny were screened for the presence of homozygous recessive mutations. One such recovered mutation, *gimpy*, is described here. Affected embryos are shorter than their sibs, have aberrant brain morphology and lack a differentiated notochord - thus, they resemble the 'dwarf' class of mutants recovered in other mutagenesis screens. I report the analysis of this mutant using the observation of embryonic morphology, *in situ* analysis and histological techniques and discuss the subsequent identification of this mutation as a knock-out of the LamininC gene.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Title</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>2</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>3</td>
</tr>
<tr>
<td>Table of contents</td>
<td>4</td>
</tr>
<tr>
<td>List of figures</td>
<td>12</td>
</tr>
</tbody>
</table>

CHAPTER 1 General Introduction 14

PART I The Rho Family of Small GTPases 14

1.1.1 General features of the Rho family 14
  1.1.1.1 Identification of Rho family members 14
  1.1.1.2 Functional domains 15
  1.1.1.3 Post-translational modification 15

1.1.2 Rho family proteins mediate rearrangements of the actin cytoskeleton 16
  1.1.2.1 Rho, Rac and Cdc42 have distinct effects on cultured cells 16
  1.1.2.2 Rho GTPases regulate the morphology of neuronal cell lines 19

1.1.3 A number of factors regulate the activity of the Rho GTPases 20
  1.1.3.1 GTPase-activating proteins 20
  1.1.3.2 Guanine nucleotide exchange factors 21
  1.1.3.3 Guanine nucleotide dissociation inhibitors 22
  1.1.3.4 Tools for modulating Rho activities 23
    1.1.3.4.1 Constitutively active mutants 23
    1.1.3.4.2 Dominant negative mutants 23
    1.1.3.4.3 The C3 family of exoenzymes 23
    1.1.3.4.4 Other bacterial toxins 25

1.1.4 Rho family members act downstream via interacting effector proteins 25
  1.1.4.1 Rho effectors mediating cytoskeletal rearrangements 28
  1.1.4.2 Rac and Cdc42 effectors mediating cytoskeletal rearrangements 30
  1.1.4.3 Rho family GTPases activate several transcriptional pathways 32

1.1.5 Other cellular activities involving the Rho family 33
  1.1.5.1 Cell adhesion 33
  1.1.5.2 Endocytosis and secretion 35
  1.1.5.3 Phagocytosis and the oxidative response 36
  1.1.5.4 Cytokinesis 37

1.1.6 Rho GTPases are required in developmental processes in vivo 38
  1.1.6.1 Expression patterns in a number of organisms suggest a requirement during embryogenesis 38
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1.6.1.1 Early embryogenesis in <em>Drosophila</em> requires Rho, Rac and Cdc42</td>
<td>38</td>
</tr>
<tr>
<td>1.1.6.1.2 Drosophila dorsal closure requires Rho GTPases</td>
<td>39</td>
</tr>
<tr>
<td>1.1.6.1.3 Maternal and zygotic Rho1 phenotypes differ in <em>Drosophila</em> embryos</td>
<td>41</td>
</tr>
<tr>
<td>1.1.6.2 The generation of epithelial planar polarity requires Rho family function</td>
<td>42</td>
</tr>
<tr>
<td>1.1.6.2.1 <em>Drosophila</em> epithelial polarity</td>
<td>42</td>
</tr>
<tr>
<td>1.1.6.2.2 Hair cell morphogenesis</td>
<td>44</td>
</tr>
<tr>
<td>1.1.6.3 Rho family proteins are required during axonogenesis and dendritic branching</td>
<td>45</td>
</tr>
<tr>
<td>1.1.6.3.1 Neuritogenesis and the growth cone</td>
<td>45</td>
</tr>
<tr>
<td>1.1.6.3.2 Rho GTPases are required for dendritic morphology</td>
<td>46</td>
</tr>
<tr>
<td>1.1.6.3.3 A neural specific Rac induces neuritogenesis in the chick</td>
<td>48</td>
</tr>
<tr>
<td>1.1.6.3.4 Upstream signals are required for GTPase activation</td>
<td>48</td>
</tr>
<tr>
<td>1.1.6.3.5 RhoA is required for nervous system development in <em>Drosophila</em></td>
<td>49</td>
</tr>
<tr>
<td>1.1.6.3.6 Drac1 is required for sensory and motor neuron guidance in <em>Drosophila</em></td>
<td>50</td>
</tr>
<tr>
<td>1.1.6.3.7 The role of other GTPase family members in the nervous system</td>
<td>51</td>
</tr>
<tr>
<td>1.1.6.4 RhoB and neural crest formation</td>
<td>52</td>
</tr>
<tr>
<td>1.1.6.5 Rho GTPases and muscle development</td>
<td>52</td>
</tr>
<tr>
<td>1.1.7 Clinical perspectives</td>
<td>53</td>
</tr>
<tr>
<td>1.1.7.1 Rho GTPases and the immune system</td>
<td>53</td>
</tr>
<tr>
<td>1.1.7.1.1 Rac mediates neutrophilic responses</td>
<td>53</td>
</tr>
<tr>
<td>1.1.7.1.2 A role for Rho in the thymus</td>
<td>54</td>
</tr>
<tr>
<td>1.1.7.2 Rho GTPases and the circulatory system</td>
<td>55</td>
</tr>
<tr>
<td>1.1.7.2.1 Rho is involved in angiogenesis</td>
<td>55</td>
</tr>
<tr>
<td>1.1.7.2.2 Alteration of Rho expression leads to heart failure</td>
<td>55</td>
</tr>
<tr>
<td>1.1.7.2.3 Rac contributes to post-ischaemic reperfusion injury</td>
<td>56</td>
</tr>
<tr>
<td>1.1.7.3 Rho GTPases and cancer</td>
<td>57</td>
</tr>
<tr>
<td>1.1.7.3.1 Rho family members may be implicated in oncogenic transformation</td>
<td>57</td>
</tr>
<tr>
<td>1.1.7.3.2 Rho and hamartoma formation</td>
<td>57</td>
</tr>
<tr>
<td>1.1.7.3.3 RhoC may be required for metastasis</td>
<td>58</td>
</tr>
<tr>
<td>1.1.7.4 Wound healing</td>
<td>58</td>
</tr>
<tr>
<td>1.1.7.4.1 Rho GTPases are necessary for embryonic wound healing</td>
<td>58</td>
</tr>
</tbody>
</table>
Table of contents

1.I.7.4.2 Rho regulates platelet aggregation 60
1.I.8 Summary 61

PART II The Zebrafish as a Developmental Model 62
1.II.1 The rise of the zebrafish 62
  1.II.1.1 The development of the zebrafish embryo 62
    1.II.1.1.1 The development of the blastula 62
    1.II.1.1.2 Epiboly and gastrulation 65
    1.II.1.1.3 Axis specification and tissue restriction 66
    1.II.1.1.4 Segmentation stages 67
    1.II.1.1.5 Pharyngula and beyond 68
  1.II.1.2 Mutagenesis screening 69
  1.II.1.3 Future directions for the zebrafish 72
1.II.2 Summary 73
1.2 The project 74

CHAPTER 2 Materials and Methods 75
2.1 Maintenance of Zebrafish 75
2.2 Observation of Live Embryos 75
2.3 Molecular Biology Techniques 75
  2.3.1 cDNA cloning of zebrafish small GTPases 76
    2.3.1.1 Cloning of zebrafish RhoA 76
    2.3.1.2 Cloning of zebrafish Rac1 76
    2.3.1.3 Nomenclature 77
  2.3.2 Southern Blotting 77
2.4 In Situ Hybridisation 78
  2.4.1 Synthesis of antisense RNA probes for in situ hybridisation 78
  2.4.2 Single whole-mount in situ hybridisation 78
    2.4.3 In situ hybridisation of adult brain tissue 79
    2.4.4 cDNAs used for in situ hybridisation 80
    2.4.5 Cryosectioning of stained embryos 80
2.5 Northern Blotting 80
  2.5.1 Total RNA extraction 80
  2.5.2 Northern Blotting 81
    2.5.2.1 Preparation of the gel and samples 81
    2.5.2.2 Transfer of RNA onto and hybridisation of the filters 81
2.6 Injection of mRNA into Early Stage embryos 82
  2.6.1 Cloning of hRho and C3 constructs into expression vectors 82
  2.6.2 Other templates used for RNA synthesis 82
Table of contents

2.6.3 In vitro transcription of mRNA for injection 83
2.6.4 Injection of mRNA into early stage embryos 83
2.7 Analysis of GFP expression 83
2.8 Immunohistochemistry on whole-mount embryos 84
  2.8.1 Fixation of tissue 84
  2.8.2 Labelling 84
2.9 Haematoxylin staining 85
2.10 Acridine orange staining 85
2.11 Alcian Blue staining 85
2.12 Electron microscopy 86
2.13 ENU Mutagenesis Screen 86
  2.13.1 ENU mutagenesis 86
  2.13.2 Mutant analysis 87

CHAPTER THREE The Cloning and Expression of Two Members of the Zebrafish Rho GTPase Family 88
3.1 Introduction 88
3.2 Results 88
  3.2.1 Cloning of zebrafish zRhoA 88
    3.2.1.1 Isolation of a zRhoA clone 88
    3.2.1.2 Evolutionary comparisons with RhoA from other species 91
  3.2.2 Expression of zRhoA during development 91
    3.2.2.1 Expression during early embryogenesis 91
    3.2.2.2 Expression during segmentation stages 91
    3.2.2.3 Expression during pharyngula and hatching-period stages 96
  3.2.3 Cloning of a partial zebrafish zRac1 fragment 101
    3.2.3.1 Isolation of a zRac1 clone 101
    3.2.3.2 Evolutionary comparisons with Rac1 from other species 101
  3.2.4 Expression of zRac1 during development 106
    3.2.4.1 Expression during early embryogenesis 106
    3.2.4.2 Expression during segmentation stages 106
    3.2.4.3 Expression during pharyngula and hatching-period stages 106
  3.2.5 Northern blot analysis 113
    3.2.5.1 zRhoA expression 118
    3.2.5.2 zRac1 expression 118
  3.2.6 Expression of zRhoA and zRac1 in adult zebrafish brain 118
    3.2.6.1 zRhoA expression 118
    3.2.6.2 zRac1 expression 121
    3.2.6.3 Haematoxylin staining 121
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3 Discussion</td>
<td>121</td>
</tr>
<tr>
<td>3.3.1 Rho GTPase family homologues in the zebrafish</td>
<td>121</td>
</tr>
<tr>
<td>3.3.2 Evolutionary relationships between zebrafish Rho GTPases and those from other species</td>
<td>122</td>
</tr>
<tr>
<td>3.3.2.1 zRhoA</td>
<td>122</td>
</tr>
<tr>
<td>3.3.2.2 zRacl</td>
<td>123</td>
</tr>
<tr>
<td>3.3.3 Comparison of expression patterns in the zebrafish with those in other species</td>
<td>124</td>
</tr>
<tr>
<td>3.3.3.1 Expression of RhoA</td>
<td>124</td>
</tr>
<tr>
<td>3.3.3.2 Expression of zRacl</td>
<td>126</td>
</tr>
<tr>
<td>3.3.4 Northern blot analysis</td>
<td>127</td>
</tr>
<tr>
<td>3.3.5 Rho family expression in developing and mature brain tissue</td>
<td>128</td>
</tr>
<tr>
<td>3.4 Conclusions</td>
<td>128</td>
</tr>
</tbody>
</table>

CHAPTER FOUR An Investigation into the Role of the RhoA GTPase During Zebrafish Embryogenesis | 129 |
| 4.1 Introduction | 129 |
| 4.2 Results | 130 |
| 4.2.1 Synthetic RNA is translated efficiently in vivo | 130 |
| 4.2.2 Overexpression of wild-type or dominant negative Rho has no effect on embryogenesis | 130 |
| 4.2.3 Injection of constitutively active V14RhoA | 133 |
| 4.2.3.1 Injection of constitutively active V14RhoA produces an epiboly phenotype that is dose-dependent | 133 |
| 4.2.3.2 V14Rho injected embryos are abnormal during epiboly and have a shortened axis | 134 |
| 4.2.3.3 Injected embryos show errant expression of anterior and axial markers | 137 |
| 4.2.3.4 Early brain specification is altered in injected embryos | 140 |
| 4.2.3.5 Posterior mesoderm is positioned incorrectly in injected embryos | 140 |
| 4.2.3.6 V14Rho injected embryos have anterior patterning defects later in development | 145 |
| 4.2.3.7 The morphological defects are mirrored by disrupted gene expression patterns in the brain and eye | 145 |
| 4.2.3.8 V14Rho generates patterning defects in the CNS | 152 |
| 4.2.3.9 Injected embryos have cranio-facial defects | 152 |
4.2.3.10 V14Rho injection phenocopies the silberblick mutation

4.2.4 Injection of C3 exoenzyme RNA

4.2.4.1 C3 RNA is functional at very low doses

4.2.4.2 C3 RNA produces a phenotype shortly after injection

4.2.4.3 C3 does not block gene transcription in injected embryos

4.2.4.4. C3 injected embryos are capable of nuclear division

4.3 Discussion

4.3.1 Overexpression of wild-type or dominant negative RhoA has no effect on zebrafish embryogenesis

4.3.1.1 Injected zebrafish embryos are indistinguishable from wild-type sibs

4.3.1.2 Overexpression of these constructs in Drosophila blocks cellularisation and dorsal closure

4.3.1.3 Modifying expression of RhoA leads to epithelial polarity defects

4.3.1.4 There is no clear reason for the lack of phenotype seen in zebrafish

4.3.2 Expression of constitutively active RhoA causes fusion of the eyes and aberrant brain morphology

4.3.2.1 V14Rho brings about an obvious epiboly phenotype

4.3.2.2 This phenotype is unlike that seen in other organisms

4.3.2.3 At later stages V14Rho injected embryos have fused eyes and aberrant brain morphology

4.3.2.4 Cyclopia is generated by incomplete partitioning of the eye field, as seen in the silberblick mutant

4.3.2.5 Interfering with the non-canonical Wnt pathway gives a similar phenotype

4.3.3 Blocking all Rho function has a severe effect on embryogenesis

4.3.3.1 C3 exoenzyme acts within minutes of being transcribed

4.3.3.2 C3 does not prevent gene transcription

4.4 Conclusions

CHAPTER FIVE The characterisation of gimpy, a mutation recovered from an ENU mutagenesis screen

5.1 Introduction

5.2 Results

5.2.1 Identification of the gimpy mutation
5.2.2 Embryonic morphology
  5.2.2.1 Live examination of embryos reveals a number of defects
  5.2.2.2 The ultrastructure of the notochord is severely disrupted

5.2.3 Patterning of the central nervous system
  5.2.3.1 Immunostaining for anti-acetylated tubulin reveals severe defects in the axon scaffold and the embryonic brain
  5.2.3.2 The Mauthner neurons in the hindbrain fails to project correctly
  5.2.3.3 In situ analysis reveals neural gene expression to be largely wild-type

5.2.4 Patterning of the eye
  5.2.4.1 Immunostaining reveals defects in the projection of the optic nerve and the layering of the retina
  5.2.4.2 Expression of \textit{pax2.1} is not abnormal

5.2.5 The expression of \textit{sonic hedgehog} suggests a signalling capacity in the undifferentiated notochord tissue

5.2.6 By 48hpf \textit{gimpy} mutants become paralysed
  5.2.6.1 Motor neurons project aberrantly within the somites
  5.2.6.2 The distribution of myosin is defective in \textit{gimpy} muscles
  5.2.6.3 The ultrastructure of the somite reveals mistargetting of myofibrils

5.2.7 \textit{gimpy} embryos have an aberrant body growth phenotype
  5.2.7.1 The pituitary gland develops normally in \textit{gimpy} embryos
  5.2.7.2 The reduced body length does not result from an increase in cell death
  5.2.7.3 \textit{gimpy} embryos have severe craniofacial defects

5.2.8 The ultrastructure of the skin is greatly disrupted

5.3 Discussion
  5.3.1 Morphological defects of the \textit{gimpy} mutant
  5.3.2 The scaffold of the central nervous system is severely disrupted in \textit{gimpy}
  5.3.3 The prepatter of the central nervous system appears to be normal
  5.3.4 There is a mild midline defect in \textit{gimpy}
  5.3.5 The paralysis phenotype does not have a clear cause
5.3.6 Ultrastructural examination reveals a complete collapse of the notochord 225
5.3.7 The lack of a basal lamina gives clues as to the nature of the mutation 226
5.3.8 Identification of the *gimpy* locus 226
5.3.9 Cloning of *sleepy, bashful* and *grumpy* 227

5.4 Conclusions 229

CHAPTER SIX General Discussion and Future Directions 230
6.1 Rho family GTPases in the zebrafish 230
   6.1.1 *zRhoA* and *zRac1* - the isolation of new family members 230
   6.1.2 Searching for other Rho family members 231
   6.1.3 Functional assays 232
   6.1.4 Summary 234
6.2 Dwarf-like mutants in the zebrafish 235
   6.2.1 Recovery of laminin mutations from mutagenesis screens 235
   6.2.2 Summary 236

CHAPTER SEVEN Bibliography 237

Appendix 1 262
Appendix 2 264
Appendix 3 265
Appendix 4 266
List of Abbreviations 267
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>GTPase cascades in fibroblasts</td>
<td>18</td>
</tr>
<tr>
<td>1.2</td>
<td>General model for activation of effector proteins by Rho GTPases</td>
<td>27</td>
</tr>
<tr>
<td>1.3</td>
<td>Camera lucida drawings of the zebrafish embryo at various stages</td>
<td>64</td>
</tr>
<tr>
<td>1.4</td>
<td>Mutagenesis screening produces homozygous mutant zebrafish embryos</td>
<td>71</td>
</tr>
<tr>
<td>3.1</td>
<td>Nucleotide sequence and deduced amino acid sequence of the zebrafish zRhoA clone</td>
<td>90</td>
</tr>
<tr>
<td>3.2</td>
<td>ClustalW alignment of zRhoA peptide sequence against RhoA proteins from other species</td>
<td>93</td>
</tr>
<tr>
<td>3.3</td>
<td>The expression of zRhoA during zebrafish embryogenesis</td>
<td>95</td>
</tr>
<tr>
<td>3.4</td>
<td>Closer examination at later stages of development reveals more clearly defined domains of zRhoA expression</td>
<td>98</td>
</tr>
<tr>
<td>3.5</td>
<td>At progressive timepoints during development, zRhoA is expressed in different regions of the trunk</td>
<td>100</td>
</tr>
<tr>
<td>3.6</td>
<td>Nucleotide sequence and deduced peptide sequence for the zRac1 coding region</td>
<td>103</td>
</tr>
<tr>
<td>3.7</td>
<td>ClustalW alignment of zRac1 peptide sequence against Rac1 sequences from other species</td>
<td>105</td>
</tr>
<tr>
<td>3.8</td>
<td>The expression of zRac1 during zebrafish embryogenesis</td>
<td>108</td>
</tr>
<tr>
<td>3.9</td>
<td>Subtleties in the zRac1 expression pattern become apparent later in development</td>
<td>110</td>
</tr>
<tr>
<td>3.10</td>
<td>At progressive timepoints during development, zRac1 is expressed in different regions of the trunk</td>
<td>112</td>
</tr>
<tr>
<td>3.11</td>
<td>Total RNA extraction and Northern blot analysis for zRhoA at different developmental stages</td>
<td>115</td>
</tr>
<tr>
<td>3.12</td>
<td>Total RNA extraction and Northern blot analysis for zRac1 at different developmental stages</td>
<td>117</td>
</tr>
<tr>
<td>3.13</td>
<td>Adult brain sections reveal different expression patterns for zRhoA and zRac1</td>
<td>120</td>
</tr>
<tr>
<td>4.1</td>
<td>Staining for the Myc epitope shows the expression of RNA injected at the 1-4 cell stage in the mid-gastrulation embryo</td>
<td>132</td>
</tr>
<tr>
<td>4.2</td>
<td>V14Rho RNA expression produces a variable epiboly phenotype</td>
<td>136</td>
</tr>
<tr>
<td>4.3</td>
<td>Expression of axial and anterior markers at tailbud stage in V14Rho RNA injected embryos</td>
<td>139</td>
</tr>
<tr>
<td>4.4</td>
<td>Expression of pax2 shows a disrupted presumptive midbrain-hindbrain boundary at tailbud stage</td>
<td>142</td>
</tr>
<tr>
<td>4.5</td>
<td>Expression of posterior mesoderm markers in V14Rho RNA injected embryos</td>
<td>144</td>
</tr>
</tbody>
</table>
4.6 Injection of 50ng/μl V14Rho RNA has a severe affect on the patterning of the anterior region of the embryo 147
4.7 Expression of pax2.1 in injected embryos at 18-20 somites 149
4.8 Expression of pax6 at 18-20 somites 151
4.9 Later in embryogenesis, V14Rho’s effects on facial morphology become more pronounced 154
4.10 Staining for anti-acetylated tubulin at 48hpf reveals neural defects in embryos injected with 50 ng/μl V14Rho RNA 156
4.11 Homozygous slb embryos have a phenotype similar to V14Rho RNA injected embryos 158
4.12 Injection of C3 RNA produces a phenotype within a few hours of injection 161
4.13 Time-lapse series showing development of a wild-type control embryo 163
4.14 Time-lapse series showing development of a C3 RNA injected embryo 165
4.15 Injection of C3 RNA has no effect on gene transcription per se 168
4.16 Expression of P-H3, a marker of dividing cells, is downregulated in C3 RNA injected embryos 170
5.1 The gimpy mutation becomes discernible by 24hpf 188
5.2 The primary morphological defects in the gimpy mutant at 24hpf 190
5.3 gimpy mutants have a disorganised notochord at the ultrastructural level 193
5.4 Immunostaining using anti-acetylated tubulin shows defects in the developing CNS at 48hpf 195
5.5 Immunostaining using the 3A10 antibody at 26hpf 198
5.6 Krox20 expression at 26hpf reveals hindbrain morphology defects in gimpy 200
5.7 The zn-5 antibody shows severe defects in the eye and aberrant staining in the trunk at 48hpf 203
5.8 Expression of pax2 in gimpy embryos at 24hpf 205
5.9 sonic hedgehog expression is normal in gimpy mutant embryos at 26-28hpf 208
5.10 Immunostaining using the pan-myosin antibody reveals severe muscular defects 211
5.11 Anterior-posterior muscle patterning is disrupted in gimpy at 48hpf 214
5.12 Acridine Orange staining reveals normal levels of cell death in gimpy at 24hpf 217
5.13 Craniofacial defects in gimpy shown by Alcian Blue staining at 5dpf 219
5.14 Transmission electron micrographs of wild-type and gimpy mutant periderm at 48hpf 222
CHAPTER ONE

General Introduction

The work in this thesis can be broadly divided into two sections. The first section looks at the role of the Rho family of small GTPases during vertebrate embryogenesis, using the zebrafish as a model organism. The second section deals with the characterisation of gimpy, a dwarf-type mutant arising from a zebrafish ENU mutagenesis screen.

This introduction is divided accordingly, with the first section reviewing the Rho family of GTPases, and the second surveying the use of the zebrafish as an experimental model, with reference to the cellular and morphogenetic events known to take place during early development.

PART I

The Rho Family of Small GTPases

1.1.1 General features of the Rho family

The Rho family of small G proteins is a sub-group of the Ras superfamily, a set of 20-30kDa monomeric, GTP-binding proteins. These proteins act as 'molecular switches' within the cell, being active when complexed with GTP and inactive when GDP is bound. The cycling between these two states and the ratio of the two bound forms to one another is thought to be involved in the regulation of many cellular processes, particularly those involved in the remodelling of the actin cytoskeleton. A general scheme of how this is brought about is shown in Fig. 1.1.

1.1.1.1 Identification of Rho family members

The first Rho family member to be identified was isolated from the sea-slug, Aplysia californica and was so named on account of its Ras homology (Anderson & Lacal, 1987). Since then, numerous family members, often with multiple isoforms, have been identified from all the eukaryotic species so far investigated including Drosophila and Saccharomyces cerevisiae (Luo et al., 1994; Madaule et al., 1987) - indeed, there are at least ten different mammalian Rho GTPases: Rho (A, B, C isoforms), Rac (1, 2, 3 isoforms), Cdc42 (Cdc42Hs, G25K isoforms), Rnd1/Rho6, Rnd2/Rho7, Rnd3/RhoE, RhoD, RhoG, TC10, TTF (Bishop & Hall, 2000). At the
proteins, the amino acid sequences of the different family members are highly homologous, both within and between species - for example, human RhoA and RhoC are 92% identical to each other and both are 85% identical to RhoB; whereas human Rac1 and chick Rac1a are 100% identical (Malosio et al., 1997; Ridley, 1997). In general, the majority of differences occur in the C-terminal portion of the protein - the N-terminal sequences tend to be more conserved.

1.1.1.2 Functional domains

The generic family member, RhoA, is 193aa long and contains five regions required for the correct functioning and localisation of the protein, four of which are highly conserved between Ras superfamily members. Broadly, these domains are GxxxxGKS/T at positions 13-20; DxxG at 59-62; NKxD at 117-120; and ExSA at 158-161 (x is denotes any amino acid) and some are required for GTP or Mg$^{2+}$ binding, others for GTP hydrolysis. The fifth and final region is the C-terminal C-A-A-X box, (where A denotes an aliphatic, non-polar residue), a cysteine motif that is required for \textit{in vivo} co-valent modification (Ridley, 1997; Valencia et al., 1991). As mentioned above, these functional domains are common to Ras superfamily GTPases and the crystal structures of Ras, Rho and Rac are similar. However, some differences are thought to exist, such that in the Rho family, there is an insert region centred around position 127. This region is highly variable in sequence when Rho, Rac and Cdc42 are compared, suggesting that this region mediates a specificity response based on interactions with separate sets of factors - for example, in Rac1, this region is thought to be involved in the induction of NADPH oxidase activation (Ridley, 1999). RhoA, B and C also possess a basic insert, Arg5 Lys6, which is one of the sequences required for modification of these proteins by the clostridial enzyme, C3 transferase (Wilde et al., 2000).

1.1.1.3 Post-translational modification

As mentioned above, there is a conserved C-terminal C-A-A-X motif in many members of the Ras superfamily, particularly in the Rho groups. This region is required for a sequence of events leading to the correct subcellular localisation of the proteins. The first step is an isoprenylation reaction at the conserved cysteine residue. Following this, it is carboxymethylated and the A-A-X triplet is cleaved proteolytically. These modifications are thought to be necessary for the functioning of the proteins (Adamson et al., 1992a), as RhoA and RhoC are principally cytosolic but a small fraction is found at the plasma membrane which is its site of action. Likewise, RhoB has to be targeted precisely to early endosomal and pre-lysosomal
vesicles - in either case, the addition of a lipid moiety to the protein would help to increase hydrophobicity for membrane association (Adamson et al., 1992b) and the prenyl group is also thought to be required for interaction with inhibitory factors. In addition, RhoA is also said to be phosphorylated on Ser188, which may act to regulate its function at some level (Ridley, 1999).

1. Introduction

1.1.2 Rho family proteins mediate rearrangements of the actin cytoskeleton

1.1.2.1 *Rho, Rac and Cdc42 have distinct effects on cultured cells*

A series of classic experiments demonstrated the effects of the Rho GTPases on the cytoskeleton and morphology of cultured cells, with the Swiss 3T3 fibroblast being used as a paradigm in many of these investigations. Filamentous actin in the cell is generally organised into three types of structure: stress fibres, long actin cables which cross the cell and mediate attachment to the external environment via focal adhesions; a dense network of filaments found at the leading edge of cells, producing membrane ruffles or lamellipodia; and shorter bundles which protrude through the membrane to form filopodia. The formation of all of these elements can be induced by the activation of endogenous GTPases, or by ectopic introduction of activated mutant forms.

Extracellular growth factors are key regulators of actin organisation. In Swiss 3T3 cells, serum-starvation induces the cells to become quiescent, such that they lose actin protrusions and focal adhesions. Addition of serum or its active component lysophosphatidic acid (LPA) or the factor bombesin gives a rapid response - within minutes, an array of stress fibres forms, anchored to the extracellular substratum via integrin-based focal adhesions (Ridley & Hall, 1992). It was found that constitutively active Rho induced these effects in the absence of LPA, whereas prior treatment of the cells with C3 exoenzyme to inhibit Rho function prevented any serum-mediated changes. Thus, it was proposed that a Rho-regulated pathway transduces the LPA-stimulated cellular response. These effects were not limited to 3T3 cells; for example, Rho was also found to mediate the motility and bone-resorption response in osteoclasts, via osteopontin-stimulated podosome formation (Chellaiah et al., 2000).

The addition of other growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF) or insulin to 3T3 cells was found to induce the formation of membrane ruffling and, as a delayed response, stress fibres. Again, these structures could be inhibited by the injection of dominant negative Rac, or
Fig. 1.1 GTPase cascades in fibroblasts

Various extracellular stimuli trigger the activation of small GTPases, eliciting responses brought about by rearrangements of the cytoskeleton—filopodia, lamellipodia, and stress fibres may be formed. Different agonists can activate individual GTPases, but there is also thought to be cross-talk between the family members, such that Cdc42 can activate Rac, which in turn can trigger Rho.

(adapted from Van Aelst and D'Souza-Schorey, 1997)
stimulated in quiescent cells by the constitutively active form, implying the presence of a Rac-mediated pathway activated by a specific set of upstream signals. The fact that the secondary response of stress fibre formation could be inhibited by C3 exoenzyme led to the supposition that in cultured cells at least, there is cross-talk between the GTPases, such that the activation of Rac also leads to the activation of Rho (Ridley et al., 1992). However, there still remains debate about to what extent this may occur - others have found that PDGF-induced Rac (and indeed, Cdc42) activation acts to directly downregulate Rho activity and the balance between Rho and Rac activities determines cell morphology (Sander et al., 1999).

The third type of actin structure mentioned above, filopodia, are induced in 3T3 cells in response to constitutively-active Cdc42 (Nobes & Hall, 1995), accompanied by lamellipodia which often extend between two adjacent filopodia to give a 'webbed' effect. Again, there is evidence from this and from interference experiments that Cdc42 activates Rac, which in turn may or may not activate Rho. Thus, cells are able to make a coordinated response to extracellular cues, leading to increased adhesion via focal adhesions, or increased motility, due to actin-based exploratory processes.

1.1.2.2 Rho GTPases regulate the morphology of neuronal cell lines

Amongst the first investigations to be carried out into the role of Rho family proteins was the treatment of cultured rat pheochromocytoma PC-12 cells with C3 exoenzyme (Nishiki et al., 1990). Although the precise target of the enzyme was still the subject of speculation, having not been narrowed down as specifically modifying Rho at that time, the results provided a valuable early insight into the regulation of neuronal morphology - treated cells ceased to grow and instead, produced neurites, suggesting that ADP-ribosylation of either Rho or Rac relieved its inhibitory effects on process outgrowth. Further evidence for Rho being an inhibitor of neurite projection came from the observation that the collapse of the growth cone, the exploratory structure at the tip of the neurite, could be induced by microinjection of wild-type or constitutively active Rho; conversely, the induction of this collapse by LPA was be blocked by C3 treatment (Kozma et al., 1997).

Whereas Rho apparently blocks neurite extension, it was found that Rac1 and Cdc42 are required for the production of filopodia and lamellipodia in the mouse neuronal cell line N1E-115 - dominant negative versions of either blocks the formation of these structures in serum-starved cells (Kozma et al., 1997). Similarly, in PC-12 cells, Rac was found to regulate neurite outgrowth in response to treatment
1. Introduction

with nerve growth factor (Lamoureux et al., 1997). The proposed model is that Cdc42 acts upstream of Rac1 to regulate the formation of actin-rich structures in the growth cone, whereas Rho inhibits the extension of these protrusions; it is the competition between these two pathways that determines the morphology of the growth cone at a given time or location, in response to cues from the surrounding environment. *In vivo* however, there is likely to be a more subtle and dynamic balance between the activities of these two pathways. The activities of Rho GTPases in neurogenesis *in vivo* will be discussed below (1.1.6.3).

1.1.3 A number of factors regulate the activity of the Rho GTPases

The ratio of GDP- to GTP-bound forms of the Rho GTPases determines their activity level within the cell, as seen for other members of the Ras superfamily (Schmidt & Hall, 1998). Consequently, there are a wealth of interacting factors known to be involved in regulating the amount of each form present. GTPase-activating proteins (GAPs) accelerate the intrinsic hydrolytic capacity of Rho proteins, causing their inactivation; conversely, guanine nucleotide exchange factors (GEFs) trigger the exchange of bound GDP for GTP, thus 'recharging' the GTPase. Finally, the guanine nucleotide dissociation inhibitors (GDIs) associate with Rhos in resting cells and it is in these complexes that Rhos are found in the cytoplasm (Schmidt & Hall, 1998).

1.1.3.1 GTPase-activating proteins

Thus far, around 20 GAPs have been identified, with homologous proteins being found in mammals, *Drosophila*, *C. elegans* and *S. cerevisiae* (Van Aelst & D'Souza-Schorey, 1997). The proteins are characterised by the presence of a 140 amino acid catalytic domain and are variable in their specificity towards their GTPase targets - for example, the archetypal family member p50Rho-GAP shows activity towards Rho, Rac and Cdc42 *in vitro* (but has been seen to only act on Rho *in vivo* (Ridley et al., 1993)), whereas N-chimaerin shows specificity for Rac (Diekmann et al., 1991; Lancaster et al., 1994). Analysis of the crystal structures of GTPases bound to GAPs at various stages during hydrolysis reaction has revealed that the GAP acts by inserting a so-called 'arginine finger' into the GTPase active site, stabilising the 'transition' state and facilitating the inactivation of the protein.

As well as participating in the regulation of the GTPases, GAPs may also be involved in their downstream effector pathways. N- and B-chimaerin exert their GAP activities on Rac, and microinjection of the GAP domain into fibroblasts inhibited
1. Introduction

Rac- and Cdc42-induced cytoskeletal rearrangements as might be expected (Diekmann et al., 1991). However, it was also found that injection of the full-length N-chimaerin protein or a version lacking GAP activity induced the formation of lamellipodia and filopodia, implying the activation of Rac and Cdc42. Thus, it has been suggested that N-chimaerin may have an additional role as a downstream effector (Kozma et al., 1996; Lim & Michael, 1992).

1.1.3.2 Guanine nucleotide exchange factors

As stated above, GEFs catalyse the exchange of GDP for GTP on Rho proteins, thereby activating them. The mechanism by which this is occurs is via the stabilisation of the nucleotide-free state of the GTPase - thus, GDP is released and replaced immediately by GTP, as the latter is more abundant in the cell than the former. Over 30 GEFs have so far been identified, all of which share a common motif, the 200 amino acid Dbl-homology (DH) domain, named on account of its similarity to the Dbl oncogene product which was found to induce tumorigenicity in 3T3 cells (Cerione & Zheng, 1996; Eva & Aaronson, 1985). Although this DH domain is known to be necessary and sufficient for the GTP exchange and the tumorigenic potential (Cerione & Zheng, 1996; Van Aelst & D’Souza-Schorey, 1997), an additional pleckstrin homology (PH) domain is found C-terminally adjacent to the DH domain and is thought to facilitate membrane localisation via its mediation of protein-protein or protein-lipid interactions (Schmidt & Hall, 1998). It has also been shown for many mammalian GEFs that mutation in or loss of the PH domain results in a loss of transforming capacity. This can be restored by the addition of a C-terminal C-A-A-X domain which mediates a lipid-based covalent modification and targeting to the membrane (Hori et al., 1991; Zheng et al., 1996).

Most of the GEFs identified have been seen to have oncogenic capacity in transfection experiments and this ability is often accompanied \textit{in vivo} by N-terminal truncations of the wild-type protein which spare the DH and PH domains, the suggestion being that the N-terminus has some sort of auto-inhibitory function (Schmidt & Hall, 1998). Although the assumption has been made that the presence of the tandem DH and PH domains qualify a protein as being a GEF, this is not necessarily the case - 'orphan' proteins such as Tim and Dbs possess this motif and are oncogenic, yet do not seem to act on any GTPases (Van Aelst & D’Souza-Schorey, 1997). Similar to GAPs, the true GEFs vary in their specificity towards their substrates: for instance, Vav functions as a GEF for Rho, Rac and Cdc42, whereas Lbc acts on Rho alone (Crespo et al., 1997; Zheng et al., 1995). \textit{In vivo} microinjection assays have demonstrated that the GEFs function as predicted from
the *in vitro* exchange data - Lbc induces stress fibre formation, confirming its action on Rho; likewise, the Fgd1 protein, which is required for embryonic skeletal development (Pasteris et al., 1994), causes the formation of filopodia, as predicted from its *in vitro* activity on Cdc42 (Olson et al., 1996). As well as their effects on the actin cytoskeleton, there is also evidence to suggest that GEFs can mediate the signalling pathways downstream of GTPase activation, as judged by the triggering of the JNK pathway by Dbl and Vav (Olson et al., 1996).

Aside from the defining DH and PH domains, there is little structural similarity between the various GEFs, and the profusion of different functional domains found amongst them suggests various means by which they themselves might be stimulated by upstream factors, and how they may participate in signalling cascades. Many possess SH2 or SH3 (src homology) domains, kinase domains, sites for calmodulin binding (IQ domains) or even, in the case of Bcr, a RacGAP activity (Schmidt & Hall, 1998; Van Aelst & D'Souza-Schorey, 1997). Although little is currently known about the activation of GEFs by upstream factors, there is evidence to suggest that phospholipids in the cell may act on some of their number: phosphotidylinositol 4,5-bisphosphate (PIP\(_2\)) binds to the PH domain of Vav and acts as an inhibitor, whereas the product of PI 3-kinase acting on this substrate, phosphotidylinositol 3,4,5-triphosphate (PI(3, 4, 5)P\(_3\)), binds more strongly at the PH domain and stimulates Vav's GEF activity (Han et al., 1998). PDGF is known to stimulate PI 3-kinase activation and thus the membrane ruffling seen as a response to PDGF treatment may well result from the binding of PI(3, 4, 5)P\(_3\) to RacGEFs and their concomitant stimulation (Ridley et al., 1992).

1.1.3.3 Guanine nucleotide dissociation inhibitors

The GDIs remain the least well-characterised of the factors regulating GTPase activity. In resting cells, GTPases are complexed with GDIs in the cytoplasm, to be released and translocated to the membrane when activation occurs. Rho is thought to be released when the Rho/RhoGDI complex binds to the N-terminal region of an ERM(ezrin/radixin/moesin) family member at the periphery of the cell (Hirao et al., 1996). GDIs can also solubilise and 'mop up' GTPases from the membrane (Lang et al., 1996); therefore, GDIs may be key figures in regulating the ratio of active:inactive GTPases in the cell (Schmidt & Hall, 1998).
1. Introduction

1.1.3.4 Tools for modulating Rho activities

1.1.3.4.1 Constitutively active mutants

The isolation of the Rho gene family was originally driven by the search for genes similar to Ras, which had been discovered earlier on account of its highly oncogenic potential - three isoforms of Ras, namely H-Ras, K-Ras and N-Ras, were found to be the transforming agent in many human and animal tumours. In 10-50% of human tumours, a point mutation leading to an amino acid substitution at positions 12 or 61 was found to have constitutively activated one of these genes, imbuing it with oncogenic ability (Valencia et al., 1991). Although not as clearly implicated in oncogenicity as their relatives, substitutions of Val for Gly at position 14, or Leu for Glutamine at position 63 (Rho numbering) generates similarly activated forms of the Rho GTPases caused, as for Ras, by preventing both the low-level of intrinsic GTPase activity and the accelerated level brought about by interaction with GTPase-activating proteins (GAPs). It is interesting to note that the Rnd1-3 and TTF Rho family members lack these supposedly critical residues at positions 14 and 63 and are thought not to bind GDP nor undertake the GTP hydrolysis reaction, rendering them constitutively active (Bishop & Hall, 2000; Ridley, 1999).

1.1.3.4.2 Dominant negative mutants

Similarly, dominant negative forms of these proteins can also be generated by a single amino acid substitution. A conversion of Asn to Thr at position 19 in Rho (17 in Rac or Cdc42) gives rise to a protein that can bind to the relevant nucleotide exchange factors but is unable to generate a productive response. Hence, introduction of these constructs into cells has a dominant negative effect by competing with endogenous Rhos for binding to these factors (Feig, 1999).

1.1.3.4.3 The C3 family of exoenzymes

A key tool in the research into Rho's functioning has been the C3 exoenzyme, a 25kDa protein isolated from the C and D strains of Clostridium botulinum. It was noted that when Swiss 3T3 cells were treated with this toxin, the cell bodies would round up and binucleate cells would accumulate, suggesting an effect on the cytoskeleton and an inhibition of cytokinesis (Rubin et al., 1988). The substrate in question was found to be a 22kDa protein and was soon identified to be the Rho members of the Rho subfamily (Aktories et al., 1989; Chardin et al., 1989; Quilliam...
et al., 1989) - there is still debate as to whether Rac and Cdc42 are also substrates, with the former being modified to a minor extent in the presence of special conditions, such as a low concentration of SDS (Wilde et al., 2000).

C3 and the related epidermal differentiation inhibitor (EDIN) toxins from *Bacillus cereus* and *Staph. aureus* lack membrane binding or translocation domains and thus enter cells by pinocytosis. They exert their actions by ADP-ribosylation of the Asn41 residue present in Rho (Sekine et al., 1989). This confined substrate specificity is surprising, given the presence of the same residue at position 39 in Rac and Cdc42 and considerable homology in the flanking regions. ADP-ribosylation has no significant effects on GTPase activity either intrinsic or GAP-mediated, but it blocks Rho functioning by decreasing the activity of the nucleotide exchange factor Lbc and also by inhibiting the RhoGDI-driven cycling between the cytosol and the plasma membrane, as Rho remains sequestered in an inactive GDI complex (Wilde et al., 2000).

It would seem that all eukaryotic cells examined so far show a reactivity to C3 treatment, both *in vitro* and *in vivo*. Vero cells respond reversibly to the presence of C3 in the bathing medium, with a gradual contraction of the cell bodies and a loss of F-actin (Chardin et al., 1989). Conversely, C3 induces the projection of short neurites in the normally round PC-12 line (Rubin et al., 1988). In *Drosophila*, microinjection of C3 blocks cellularisation (Crawford et al., 1998) whereas in early *Xenopus* embryos, similar treatment prevents ingestion of the cleavage furrows (Drechsel et al., 1997) and treatment of bovine spermatozoa with C3 inhibits its motility (Hinsch et al., 1993). Substrates for C3 have also been identified in *Saccharomyces cerevisiae* and *Dictyostelium discoideum* (Bush et al., 1993; Madaule et al., 1987) and in plants - C3 affects cytoplasmic streaming in pollen tubes of the pea and in the stamen hairs of *Tradescantia*. This is a somewhat surprising, since no 'real' Rho homologues have been identified in plants - there is an abundance of Rac-like proteins. It has been posited that C3's specificity may not be as rigid as previously thought, or more likely, it is acting on an as yet unidentified plant Rho substrate (Valster et al., 2000; Yang & Watson, 1993).

The related ADP-ribosylation enzyme EDIN has also been shown to act *in vitro* and *in vivo*: in human keratinocyte culture, EDIN inhibits terminal differentiation of the cells and stimulated their growth; injection of EDIN into adult mouse skin induces epidermal hyperplasia. These events are also seen when the experiments are repeated with C3, suggesting an additional role for Rho in the regulation of the cell cycle (Sugai et al., 1992).
1. Introduction

1.3.4.4 Other bacterial toxins

Less well-characterised is the action of other toxins on Rho proteins. Toxins A and B from *Clostridium difficile* are known to glucosylate Rho, Rac and Cdc42 at Thr37 (Rho) or Thr39 (Rac, Cdc42) respectively and can lead to cell rounding followed by death (Machesky & Hall, 1996). A uropathogenic strain of *E. coli* secretes cytotoxic necrotizing factor (CNF), which catalyses the deamidation of Gln65 in RhoA, blocking its intrinsic and GAP-mediated GTPase activity and thus rendering it constitutively active (Boquet, 1999).

Deamidation at this position in RhoA can also be a consequence of exposure to *Bordetella bronchiseptica*’s dermonecrotising toxin (DNT). Acting similarly on Rac and Cdc42, DNT catalyses the cross-linking of GTPases with ubiquitous polyamines such as putrescine or spermidine, in the absence of which the aforementioned deamidation will occur. Polyamination decreases the activity of the protein without affecting its GTP-binding ability, thus again converting it into a constitutively active form - even the GDP-bound form associates fruitfully with the ROCK downstream effector. When microinjected into cultured cells, polyaminated Rho will induce stress fibres, as will the treatment of these cells with DNT alone. Hence, the modification of Rho in this way is likely to play a role in DNT’s toxic effects on cells (Masuda et al., 2000).

1.4 Rho family members act downstream via interacting effector proteins

The Rho GTPases mediate a host of downstream responses, and around 30 interacting proteins have been identified which are likely to be involved in mediating these responses at some level (Bishop & Hall, 2000). On binding GTP, the GTPases undergo a conformational change which particularly affects two regions on the surface of the GTPase, amino acids 26-45 and 59-74 (Rac numbering), which are known as switch regions I and II. The conformational differences in the GTPase are read by the effector proteins, which bind preferentially to the GTP-bound form. An additional level of binding selectivity is brought about by the amino acid sequence of switch I, such that point mutations introduced into this region can inhibit the binding of some effectors, but not others - for instance, mutating Tyr40 to Cys in Rac or Cdc42 prevents the interaction with p21-activated kinase (PAK) whereas a Phe37->Ala mutation does not; but either of these mutations inhibit Rac's interaction with mixed-lineage kinase (MLK) (Lamarche et al., 1996). However, there is a high level of amino acid sequence homology in the switch regions, entailing that they are almost identical in Rho, Rac and Cdc42. The only difference is the residue at
1. Introduction

Fig. 1.2 General model for activation of effector proteins by Rho GTPases

(1) Binding of Rho GTPase to an effector relieves an autoinhibitory intramolecular interaction.
(2) The effector remains active until GTP hydrolysis occurs.
(3) Alternatively, a modification of the effector (e.g. phosphorylation, binding a different activating protein; ellipses, Y) may maintain its activity even after dissociation of the GTPase.
(4) The effector is inactivated by removal of modification, Y, allowing the effector to re-enter its inactive state.

(adapted from Bishop and Hall, 2000)
Active effector (GTPase-independent)

Inactive effector

Rho GTPase-binding and autoinhibitory domain

Functional domain

Active effector (GTPase-dependent)
1. Introduction

position 38, being Asp in Rac and Cdc42 but Glu in Rho. This has been found to be critical for the correct binding of proteins containing CRIB (Cdc42/Rac-interactive binding) motifs, which, as may be deduced from their name, act as Rac and Cdc42 but not Rho effectors (Leonard et al., 1997).

As might be expected, the specificity of interaction with effector proteins is also somewhat dependent on regions outside the switch areas. Amino acids 143-175 in Rac have been shown, by analysis of Rho-Rac chimaeras, to be required for PAK activation, whereas the 'insert loop', amino acids 123-135, is required for Rac's interactions with the NADPH oxidase complex and the effector IQGAP but is unnecessary for PAK binding (Bishop & Hall, 2000; Diekmann et al., 1995).

Assuming that the effector proteins are inactive prior to encountering the GTP-bound GTPase, a model has been proposed for how activation might be brought about (Fig. 1.2). Functional domains in the effector are exposed due to conformational changes which relieve autoinhibitory interactions - domains mediating such an intramolecular contact have been identified in several putative effectors. Ser/Thr kinases such as the PAKs possess a domain which blocks intrinsic kinase function - when the target GTPase is bound, a conformational shift frees the inhibition and liberates the kinase domain to interact with downstream targets (Bagrodia & Cerione, 1999). Mutations in the PAK1 inhibitory domain give rise to constitutive activation of kinase activity, whereas the inhibitory domain alone can act as a dominant negative inhibitor (Zhao et al., 1998). The Rho effectors ROK and PKN are also thought to act by this inhibition-relief method. In the former case, the autoinhibitory domain actually contains the GTPase-binding region (Amano et al., 1999); for PKN though, interaction with Rho-GTP triggers autophosphorylation and lipid-binding events, both of which contribute to alleviating the inhibition which then allows activation of the kinase activity (Amano et al., 1996b).

1.4.1 Rho effectors mediating cytoskeletal rearrangements

Proteins interacting with Rho can be broadly classified into three categories, according to their binding motifs. Class I includes PKN, rhotekin and rhophilin; Class II has the coiled-coil containing ROK protein kinases; and Class III, the protein citron kinase. Although from a structural point of view, Classes I and III are homologous with respect to their Rho-binding domains (Class II proteins are strictly not homologous to either other Class), the classification is based on the distinct regions they interact with within Rho - amino acids 23-40 are required for citron;
1. Introduction

residues 75-92 for Class I proteins. The ROCKs will interact with either Rho domain (Fujisawa et al., 1998).

Of the effectors mentioned above, the function and downstream targets of several remain elusive. However, there is information emerging with respect to the intermediate steps between Rho activation and actin cytoskeleton rearrangement, with at least two effectors, ROKα and Dia, being required for the assembly of stress fibres and focal adhesions. ROKα (p164-Rho-associated kinase, or ROCK-I) and its relative ROKβ (ROCK-II, or p160ROCK) are serine/threonine kinases which increase their kinase activity and translocate to the plasma membrane when activated by Rho-GTP binding (Leung et al., 1995; Matsui et al., 1996). Introducing full-length ROKα into quiescent HeLa cells triggers stress fibre and focal adhesion assembly, whereas a kinase-dead form causes disassembly of both these structures; however, when a constitutively active catalytic domain alone is present, stellate actin-myosin filaments are generated rather than true stress fibres, suggesting that ROKα is required for actin polymerisation but that this pathway is mediated separately from that resulting in stress fibre formation (Leung et al., 1996a). Adding weight to the proposed role of ROKα in creating stress fibres is the action of Y-27632, a pharmacological inhibitor of ROKα function which prevents serum- and V14Rho-induced stress fibre formation (Uehata et al., 1997).

Downstream targets for ROKα have been identified which may well transpire to be major players in stress fibre assembly. Firstly, ROKα exhibits a kinase activity towards both myosin light chain (MLC) and the myosin-binding subunit (MBS) of MLC phosphatase. MLC phosphatase is inhibited by phosphorylation and so ROKα increases the amount of phosphorylated MLC present both directly and indirectly. This in turn enhances the assembly of actomyosin filaments and hence stress fibres are produced (Amano et al., 1996a; Bresnick, 1999). LIM kinase (LIMK) is also found to be phosphorylated by ROKα, which allows it to phosphorylate and inhibit its substrate, the actin depolymerising factor cofilin, preventing it from severing filaments and sequestering monomeric actin (Bamburg et al., 1999). ROKα also phosphorylates a ubiquitous Na+/H+ exchange factor, the activation of which helps in stress fibre and focal adhesion assembly; and adducin, which binds to F-actin when phosphorylated (Bishop & Hall, 2000). ROKβ and citron are also thought to interact with Rac, but the outcome of these events, if any, is not yet known (Schmidt & Hall, 1998).

Formin-homology proteins are also Rho effectors about which more is known, with family members including diaphanous and cappuchino in Drosophila
and Bni1p in *S. cerevisiae* (Van Aelst & D'Souza-Schorey, 1997). p140mDia, the mammalian homologue of the *Drosophila* protein, has two formin homology (FH) domains which mediate its binding to profilin, a G-actin-binding protein. By a mechanism as yet unknown, p140mDia contributes to F-actin polymerisation and organisation. Indeed, when combined with the constitutively active catalytic domain of ROKα, activated p140mDia triggers stress fibre formation and the deduction is that both are effectors required for Rho-mediated actin rearrangements (Wasserman, 1998).

There has been speculation as to the role of phospholipids in Rho-mediated effects on the cytoskeleton. As well as indirect, perhaps unrelated, effects such as the removal of capping proteins from actin filaments to generate a new crop of barbed ends onto which actin monomers can polymerise (Janmey & Stossel, 1987), there is evidence of phospholipid interaction with the Rho cascade. Antibodies to PIP$_2$ block LPA/Rho-induced effects in fibroblasts (Gilmore & Burridge, 1996); and overexpression of phosphotidylinositol 4-phosphate 5-kinase in COS-7 cells induces huge amounts of actin polymerisation, presumably by increasing the amount of PIP$_2$ present (Shibasaki et al., 1997). PIP$_2$ also may be involved in focal adhesion formation, via its ability to bind vinculin and enhance its affinity for talin, both of which are key components of the focal adhesion complex (Bishop & Hall, 2000).

1.1.4.2 *Rac* and *Cdc42* effectors mediating cytoskeletal rearrangements

Similar to the Rho effectors, downstream targets have been identified that are said to mediate Rac and Cdc42's effects on the cytoskeleton, with some of the effectors being specific to one or the other of these, other effectors acting on both.

Of the latter category, much attention has been paid to the PAK family of Ser/Thr kinases. Members of this group have been identified in mammals, *Drosophila*, *C. elegans*, fission yeast and budding yeast, where the prototype member of the family, STE20, was first found (Van Aelst & D'Souza-Schorey, 1997). STE20 was found to be involved in filamentous growth and thus was deemed to be downstream of CDC42 (Schmidt & Hall, 1998). There have been conflicting reports as to the effects of PAK activation on actin, with some finding overexpression in 3T3 or PC12 cells giving similar results to the effects of constitutively active Rac and Cdc42 (Daniels et al., 1998; Sells et al., 1997) and others finding no such effects (Lamarche et al., 1996). PAK has also been found associated with Rac/Cdc42-induced focal complexes, with its overexpression leading to focal adhesion disassembly (Schmidt & Hall, 1998). It is difficult to fully
Integrate and interpret these observations, but it is clear that PAKs play some role in Rac/Cdc42-mediated actin remodelling. This activity could be brought about by its actions on putative substrates. PAK is thought to phosphorylate and activate LIMK, suggesting a general requirement for the blocking of cofilin activity in response to GTPase stimulation; there are also reports of PAK inhibiting MLC kinase with concomitant downregulation of actomyosin filaments (Bishop & Hall, 2000). The extent to which these processes occur in vivo remains a matter of contention and could well vary in response to differing combinations of signals, in different cell types, and so on.

The PAKs contain an 18 amino acid CRIB (Cdc42/Rac-interactive binding) domain. Computer-aided searching for proteins with similar domains facilitated the identification of over 25 such proteins, again from a broad range of species (Burbelo et al., 1995). Many of the proteins found also possess kinase domains, e.g. MLK2, p120ACK, but much of the attention has been focused on the Wiskott-Aldrich syndrome protein, WASP (Aspenstrom et al., 1996). Two WASPs are known - the ubiquitous N-WASP and a WASP restricted to haemopoietic lineages. Sufferers of the disease have an X-linked immunodeficiency resulting from a loss of functional protein (Thrasher & Kinnon, 2000). WASP is a novel protein, which has several binding motifs: there is a region for binding SH2 and SH3 domains in c-fgr, a c-src family tyrosine kinase; there is a CRIB domain which expresses a preference for active Cdc42 rather than Rac; and there are also sites for binding actin monomers and the Arp2/3 complex, which acts as an actin filament nucleation site. Indeed, in rat kidney epithelial cells or Jurkat cells, overexpression of WASP brings about clustering of polymerised actin, which can be inhibited by dominant negative Cdc42 (Symons et al., 1996). Thus, it is proposed that WASP is first activated by Cdc42 and then acts as a framework upon which the components for filament assembly are brought together (Rotagi et al., 1999). A similar activity may exist for the putative Rac-interacting factor, WASP-like verprolin-homologous protein (WAVE), which also has domains for binding profilin and Arp2/3 - WAVE localises to membrane ruffles and its overexpression induces actin clustering. However, it remains to be seen whether it interacts directly with Rac in vivo (Miki et al., 1998).

Other Rac and Cdc42 effectors are known, which do not possess a CRIB domain. IQGAP1 and 2, named on account of their RasGAP homology, are thought to be complexed with Cdc42 and F-actin and may be involved with actin cross-linking (Kuroda et al., 1996). Cdc42 has specific ser/thr kinase partners, MRCKs α and β, which are thought to participate in actin rearrangements and the formation of filopodia - kinase-dead mutants block filopodia induction, whereas overexpression
of the wild-type has an opposing effect, producing enlarged filopodia (Leung et al., 1996b). Genghis Khan, the Drosophila homologue of MLCK, is involved in the cytoskeletal remodelling occurring during oogenesis (Luo et al., 1997). Rac also has its unique effectors - POR1 (Partner of Rac) was identified from a yeast two-hybrid screen and found to be involved in Rac-mediated membrane ruffling. It has been proposed that alongside its interaction with the ARF6 GTPase, POR1 might act at cell peripheries to orchestrate actin rearrangements (Van Aelst & D'Souza-Schorey, 1997). A more well-characterised target of Rac is PI 4-P5K, which raises PIP2 levels required for events such as thrombin-induced actin filament uncapping (Bishop & Hall, 2000).

1.1.43 Rho family GTPases activate several transcriptional pathways

Overexpression of Rac and Cdc42 in various cultured cell lines has been seen to stimulate the activity of the JNK and p38 MAPK signalling pathways, culminating in events at the nucleus in which transcription factors, e.g. c-Jun, Elk, are phosphorylated (Seger & Krebs, 1995). Growth factors and cytokines such as EGF and tumour necrosis factor alpha, and certain cell stresses such as heat shock and UV irradiation, are known to strongly activate these pathways - at least some of these triggers can be inhibited by dominant negative Rac and Cdc42 (Van Aelst & D'Souza-Schorey, 1997). How the GTPase are linked into these cascades still remains oblique, but there is evidence to suggest the mixed lineage kinases, MLKs, may be involved: MLKs1, 2 and 3 have CRIB motifs and can behave as MAP kinase kinase kinases (MEKKs), and kinase-dead mutants of other MEKKs blocks Rac/Cdc42 induced JNK activation (Fanger et al., 1997). However, no physiological link has yet been made between the MLKs or MEKKs and Rac or Cdc42.

Rho, Rac and Cdc42 are all known to trigger the activation of other transcriptional events. Firstly, Rho is required for serum-/LPA-induced stimulation of the serum response factor (SRF); Rac and Cdc42 can also induce SRF activation, but this is thought to be mediated by a different, Rho-independent pathway. Activation of SRF induces the formation of complexes which stimulate the transcription of genes with a serum-response element (SRE), such as c-Fos (Hill et al., 1995). The activity of SRF is thought to be regulated by actin treadmilling, with the hypothesis that it is triggered by a decrease in monomeric G-actin in the cell - overexpression of actin is found to inhibit SRF's activation (R. Treisman, unpublished). There is also mounting evidence that the Rho family are implicated in triggering the activation of NF-κB - NF-κB dimers are translocated to the nucleus after the phosphorylation-mediated release from the inhibitory I-κB they are found
complexed with, again in response to cytokines and cell stress events. Again though, the mechanism by which Rhos mediate these events is not yet clear (Perona et al., 1997).

1.1.5 Other cellular activities involving the Rho family

1.1.5.1 Cell adhesion

The regulation of cell-cell and cell-extracellular matrix (ECM) adhesion is critical for in vivo processes such as cell migration during embryogenesis, the generation of apical-basal polarity within a cell layer and the process of oncogenic transformation. Cells need to receive and interpret signals from their neighbours and environment in order to 'know' what to do next, whether to stay still, which way to move and so forth. The Rho GTPases have been implicated in the dynamic processes downstream of this signal reception, with particular emphasis on the cadherin and integrin families of cell surface receptors.

The cadherins are calcium-dependent cell-cell adhesion molecules, which are linked to the intracellular actin cytoskeleton by means of the cytoplasmic proteins α- and β-catenin, which are required for strong adhesion to occur (Kaibuchi et al., 1999): β-catenin binds the cytoplasmic tail of the cadherin and α-catenin binds to β-catenin and actin. In epithelial cells, it has been shown that the concerted action of Rho and Rac is required to modulate cadherin-based adhesion - when calcium-mediated adhesion is stimulated, actin rapidly accumulates at cell-cell contacts, a feature which can be blocked by disturbing cadherin, Rho or Rac activity (Braga et al., 1997). However, these effects may be cell-type specific, in that Rac1 and Rho are needed for E-cadherin localisation in keratinocytes, but not for VE-cadherin localisation in human umbilical vein cells (Kaibuchi et al., 1999). Cdc42 is also needed for adhesion, as is the RacGEF Tiam-1, but it is unclear whether these effects are indirect or direct. Analysis of adhesion in cells expressing chimaeric forms of E-cadherin imply that Rac1 and Cdc42 have a direct effect, whereas RhoA has more of an indirect function, mediating the rearrangement of the underlying cytoskeleton (Fukata et al., 1999; Nagafuchi et al., 1994). Rac1 and Cdc42 are thought to mediate their effects through interactions with the effector IQGAP1, which is known to bind β-catenin and thus prevent its interaction with α-catenin and the cytoskeleton. When the GTPases are activated and in the GTP-bound form at sites of cell-cell contact, they bind to IQGAP1 and relieve its inhibition on β-catenin, thus allowing reestablishment of binding to α-catenin and the cytoskeleton and strengthening cell-cell adhesions. Conversely, in the GDP-bound form, Rac1 and Cdc42 cannot bind
IQGAP1 effectively, allowing it to bind β-catenin and thus weakening cell adhesion (Kaibuchi et al., 1999). The ratio of IQGAP1 interacting with β-catenin and that bound to activated GTPases is thought to determine the adhesive potential of the cell at any given time - there are many instances where dynamic arrangement of cell-cell adhesion may be a critical physiological response. For example, MDCK epithelial cells are stimulated into motility by hepatocyte growth factor (HGF)/scatter factor, seen as the progression from membrane ruffling to a disruption of cell-cell adhesion and resulting in centrifugal cell scattering. This series of processes is thought to require the concerted efforts of Rho, Rac and Cdc42 (Hartmann et al., 1994; Kaibuchi et al., 1999). It is also interesting to note that RhoA activity can be inhibited by p120 catenin, a catenin which binds the juxtamembrane domain of cadherins - in vitro, p120 blocks GDP dissociation from the GTPase and so it is thought that binding of p120 to cadherins relieves this inhibition (Anastasiadis et al., 2000). Hence, there may exist yet another regulatory mechanism for the formation of cell-cell adhesion - it is unsurprising that cell adhesion should remain so tightly controlled, as the downregulation of cadherin function is thought to be one of the key stages in the progress of metastasis in tumour progression.

Similarly, the binding of cells to the ECM is an important regulatory event. The formation of focal adhesions upon Rho activation, or the smaller focal complexes after Rac or Cdc42 stimulation is well-observed, formed by the clustering of cell-surface integrin receptors followed by the recruitment of factors such as paxillin, talin and focal adhesion kinase (FAK) into the multimolecular complex (Hotchin & Hall, 1995). These complexes continue to recruit signalling molecules such as PI 3-kinase, MAP kinase and JNK (Miyamoto et al., 1995), and thus a pathway has been proposed which links signalling from the ECM through integrin receptors to transcriptional events in the nucleus, with accompanying cytoskeletal rearrangements.

Two mechanisms have been posited to explain how Rho may mediate these events (Keely et al., 1998). Firstly, the stimulation of PIP$_2$ production by Rho-stimulated PIP 5-kinase upregulation may promote actin polymerisation, as PIP$_2$ binds and releases actin-capping proteins such as gelsolin and profilin, allowing further nucleation to occur and the lengthening of actin filaments. PIP$_2$ also binds to the focal adhesion component vinculin, leading to a conformational change within it and allowing it to bind to talin and actin, consequently permitting the build-up of the focal complex.
1. Introduction

The second mechanism may require the activation of Rho-kinase, which itself is capable of stimulating stress fibre and focal adhesion formation. Rho-kinase brings about an increase in myosin light chain phosphorylation, which leads to the generation of a contractile force within the cell (Amano et al., 1996a). This is thought to contribute to stress fibre and focal adhesion formation - inhibition of myosin-actin contractility causes integrins to be lost from focal adhesions (Lauffenburger & Horwitz, 1996), the suggestion being that their clustering is generated from within the cell, rather than by the ECM (Burridge & Chrzanowska-Wodnicka, 1996).

1.1.5.2 Endocytosis and secretion

The regulation of endocytosis, the process by which material external to the cell or on its surface is taken up, has also been found to involve Rho family GTPases (Lamaze et al., 1996). In Xenopus oocytes, interfering with RhoA function leads to altered functions at the membrane - activation stimulates constitutive endopinocytosis, whereas C3-mediated blocking of RhoA led to the formation of membranous folds at the cell surface and prevented the uptake of sodium pumps (Schmalzing et al., 1995). Likewise, Rac has been shown to play a role in constitutive macropinocytosis in the dendritic cells of the immune system (West et al., 2000). The downstream mechanisms underlying these processes still remain oblique, though.

RhoB and RhoD have also been implicated in the internalisation of cell surface receptors. Some, such as the transferrin receptor, are recycled and return to the membrane, whereas others, such as the EGF receptor, are targeted for degradation in the lysosomes. RhoD is thought to act in regulating the rate of endosomal motility along cytoskeletal tracks, which improves the efficiency of vesicle sorting in the cell (Murphy et al., 1996). RhoD's relative RhoB is also found associated with endocytic vesicles and is has also been found to regulate the rate at which the EGF receptor is internalised and degraded (Ellis & Mellor, 2000). It has been proposed that this may present a mechanism for the maintenance of mitogenic signals. The stimulation of the EGF receptor leads to an increase in RhoB expression. As RhoB slows the progression of the receptor through the cytoplasm, this prevents it reaching the lysosome to be 'switched off' (Gampel et al., 1999). Thus the EGF signalling pathway might be regulated at an intracellular level.

Rho GTPases are also implicated in the regulation of secretory vesicle transport. In the highly-secretory mast cells, both Rho and Rac were found to be
involved in this process, which requires both outward membrane flow and the reorganisation of actin (Price et al., 1995). The downstream interactions of the GTPases in regulating secretion and endocytosis are still largely unknown, but speculation suggests the involvement of the phosphoinositide metabolism as well as a coordinated response with other GTPases such as Rab (Van Aelst & D'Souza-Schorey, 1997).

1.1.5.3 Phagocytosis and the oxidative response

Phagocytosis is a specialised form of endocytosis, whereby large particles such as microorganisms or cell debris are engulfed by specialised 'scavenger' cells such as macrophages in the bloodstream. In order to be internalised, particles must bind to the cell's surface, triggering a receptor-activated response in the cell's interior, resulting in the encapsulation of the unwanted matter within the phagosome (Aggeler & Werb, 1982). Rho family members have been implicated as being required for different stages during this process (Caron & Hall, 1998). One of the best-known triggers for phagocytosis is the presence of antibodies bound to the surface of a foreign body - the Fc receptor is stimulated by the binding to the tails of these antibodies. This induces the formation of pseudopodia which enclose the antibody-antigen complex and fuse to give the phagosome (Mellman, 1990). Rho is required for the clustering of the Fcγ receptors at the initial stage, and Rac and Cdc42 are needed for Fcγ-mediated phagocytosis, although at which stage is not yet clear (Cox et al., 1997; Ridley, 1999). All three GTPases may be required for the actin rearrangements at localised sites of the plasma membrane which occur during this process.

In macrophages, Rho, Rac and Cdc42 have been shown to be required for the co-ordination of the cytoskeletal rearrangements underlying the cellular processes such as adhesion and filopodia formation which are required for cell motility (Allen et al., 1997; Jones et al., 1998) - macrophages are necessarily motile, migrating through tissues in order to fulfil their duty. Also, to be correctly targeted to sites where they are needed, they need to be able to respond to gradients of cytokines such as CSF-1, and Cdc42 has been shown to mediate this action (Jones et al., 1998).

Rac is known to stimulate the production of superoxide radicals via the activation of NADPH oxidase (Abo et al., 1991; Diekmann et al., 1994) and these reactive oxygen species (ROS) form part of the toxic barrage directed at the ingested material. It has recently been shown in vitro how this occurs: upon the formation of the phagosome, Rac is transported to and binds phagocyte membranes, where it
induces the assembly of the NADPH oxidase complex by recruiting the p67phox subunit of the enzyme, followed by the p47phox and cytochrome b559 subunits (Gorzalczyany et al., 2000).

The entry of certain pathogenic bacteria into cells is strikingly similar to the processes seen during phagocytosis. The adherence of Salmonella or Shigella bacilli to the plasma membrane induces membrane ruffling and subsequent engulfment, similar to that seen in phagocytosis - both RhoA and Cdc42 have been implicated in this process (Lesser et al., 2000), as has Rac's involvement via the stimulation of the EGF receptor by Salmonella (Ellis & Mellor, 2000; Van Aelst & D'Souza-Schorey, 1997). Moreover, Salmonella injects a protein into its host, SopE, which acts a Cdc42 GEF, suggesting that bacteria have evolved mechanisms to subvert the normal role of small G proteins in host cells (Aktories et al., 2000; Goosney et al., 1999).

1.1.5.4 Cytokinesis

After the replication and separation of the chromosomes, cell separation occurs via the process of cytokinesis. This is accomplished by the contraction of the cleavage ring - an overlapping, circumferential ring of actin and myosin II filaments attached to the cytoplasmic face of the plasma membrane (Cao & Wang, 1990). The sliding of the filaments generates the force required for the invagination of the furrow - the contractile ring is a dynamic structure, reducing its volume by losing actin filaments as it ingresses. It is hardly surprising that a process relying upon such a wholesale rearrangement of actin involves the Rho GTPases, specifically Rho and Cdc42. In fact, Cdc42 was initially identified in S. cerevisiae as being required for bud formation and polarised cell growth (Adams et al., 1990); Rho1 mutant cells are also defective in both these processes (Yamochi et al., 1994), which require the spatio-temporal regulation of actin-containing structures and are likely to involve homologous proteins and processes as those required during cytokinesis. C3-mediated disruption of endogenous Rho proteins in dividing sand dollar eggs disrupts the actin filaments of the contractile ring without affecting nuclear division, producing multinucleate embryos (Mabuchi et al., 1993). Similarly, in Xenopus zygotes, both the inhibition and the constitutive activation of Rho or Cdc42 are found to block the ingression of the cleavage furrow, but not the specification of its position (Drechsel et al., 1997). Although the downstream targets involved in these processes are not fully elucidated, the effects seen are due to disturbing the regulation of cortical actin structures and so it could be speculated that factors such
as the Rho-regulated citron kinase are implicated (Madaule et al., 1998; Madaule et al., 2000). Likewise, the upstream regulatory factors for Rho during cytokinesis remain elusive, but may involve GEFs - it is seen that in cultured HeLa cells, a peak in the level of GTP-bound Rho is seen to occur at telophase, regulated by the exchange factor ECT2 (Kimura et al., 2000).

1.1.6 Rho GTPases are required in developmental processes in vivo

1.1.6.1 Expression patterns in a number of organisms suggest a requirement during embryogenesis

In many, if not all, of the organisms where the expression pattern of the small GTPases has been analysed, the general opinion has been that they are widely expressed during embryonic development and in adult tissues, with various degrees of spatio-temporal regulation. This topic will be dealt with in more depth in the Discussion section of Chapter 3, but to summarise, in C. elegans, D. melanogaster and the chick embryo, expression of RhoA and Rac1 homologues is reported to be more or less ubiquitous with dynamic variations in neural and mesodermal transcript levels at certain stages of development (Chen et al., 1993; Chen & Lim, 1994; Hariharan et al., 1995; Malosio et al., 1997; Sasamura et al., 1997). The widespread and uniform levels of expression would suggest a critical role for small GTPases during development and the following sections describe particular paradigms where this is the case.

A requirement for Rho GTPases during differing stages of vertebrate development has been demonstrated - for instance, RhoB is expressed in delaminating chick neural crest (Liu & Jessell, 1998), and RhoA is required for compaction in the early mouse embryo, and in the head formation pathway in Xenopus (Clayton et al., 1999; Wunnenberg-Stapleton et al., 1999). Such functions will be discussed at length in Chapter 4.

1.1.6.1.1 Early embryogenesis in Drosophila requires Rho, Rac and Cdc42

One of the earliest processes during the development of the Drosophila embryo is cellularisation, a specialised form of cytokinesis in which invaginations are driven from the plasma membrane between the cortical nuclei of the syncytial blastoderm. The invaginating furrows are actin-rich, and injection of mutant forms of Rho, Rac or Cdc42 was performed to investigate their roles in this process. Constitutively active Cdc42, dominant negative Rho and C3 exoenzyme
were the only agents found to have an effect, which was identical in each case: a disruption of the actin cytoskeleton and an aggregation of the nuclei away from the cortex, resulting in the cessation of cellularisation and ultimately, an arrest in embryogenesis (Crawford et al., 1998). Hence, the division of the syncitium into individual cells requires functioning Rho and the proper regulation of Cdc42, but perhaps surprisingly, Rac seems dispensable. It is also not known if the phenotypes seen result from effects on the cytoskeleton alone or from concerted effects with GTPase mediated signalling pathways - however, given the similarity of the effects on these embryos to the phenotypes generated by the injection of cytoskeletal-blocking agents such as cytochalasin or anti-myosin (Crawford et al., 1998), the former is likely to be the case.

Immediately after the cellularisation of the blastoderm, the process of gastrulation begins, and this too has been shown to require small GTPases, specifically Rho, for its fulfilment, (Barrett et al., 1997; Hariharan et al., 1995). During gastrulation, several morphogenetic tissue events occur, resulting in the invagination of tissue precursors such as the presumptive gut. Although the cytoskeletal processes underlying these movements are fairly well-illustrated, the signals mediating them are still relatively unknown - two signalling factors have been identified as being involved, the secreted protein folded-gastrulation (fog) (Costa et al., 1994) and the Go subunit of a heterotrimeric G protein, concertina (cta) which is thought to be linked to it (Parks & Wieschaus, 1991). A genetic screen for suppressors of a dominant negative Rho1 mutation led to the identification of DRhoGEF2, a putative exchange factor for Rho. Embryos lacking DRhoGEF2 failed to gastrulate, giving phenotypes similar to those seen for fog and cta, with aberrant cell shape changes in the cells which ought to be involuting (Barrett et al., 1997; Hacker & Perrimon, 1998). Similar defects were seen in embryos expressing dominant negative Rho, implying a specific requirement for this GTPase during gastrulation. Furthermore, DRhoGEF2 was found to be required for the morphological changes accompanying the ectopic expression of fog. Thus, the cell shape changes mediating ventral furrow formation during embryogenesis are thought to be the read-out of a signal transduction pathway triggered by the extracellular ligand fog and requiring interactions between both heterotrimeric and small G proteins.

1.1.6.1.2 *Drosophila* dorsal closure requires Rho GTPases

Dorsal closure is the process in the cells of the lateral and ventral epidermis elongate and migrate towards the dorsal midline of the embryo, thus enclosing the
embryo. During the course of these movements, there is a dynamic rearrangement of actin-based subcellular structures, such as the accumulation of actin and myosin at the leading migratory edge (Knust, 1997), and the protrusion of exploratory filopodia (Jacinto et al., 2000). Consequently, there is a requirement for Rho GTPases during this process, both for the mediation of actin rearrangements and for the signalling between cells which is needed for closure to occur correctly.

Speaking broadly, the genes involved in this process can be divided into three groups. Firstly, there are those involved in the building and maintenance of cytoskeletal elements, such as zipper, a non-muscle myosin, or α-spectrin - both of which require functioning Rac for their continued presence at the leading edge, as does actin itself (Harden et al., 1995). RhoA and its effectors such as Pkn are thought to also be needed for the maintenance of the leading edge cytoskeleton (Lu & Settleman, 1999), particularly in the cells around the segment borders, possibly by acting as a negative regulator of the transient leading-edge component losses thought to occur in these cells (Harden et al., 1999). Cdc42 has recently been shown to be essential for 'cell sampling' and the correct matching of cells across the amnioserosa during dorsal closure - at the 'zippering up' stage, cells extend long filopodia across the divide separating them, searching about until they find their correct partners. Once contact has been made, the opposing epithelial fronts are tugged towards each other and the interdigitation of the filopodia into the partner's plasma membrane results in cell fusion at the midline. Ultimately, these sites of contact are resolved into stable adherens junctions. Expression of a dominant negative Cdc42 causes only rudimentary filopodia to form and so opposite cells fail to engage and be drawn together, with misalignment of cells during the closure process. Hence, Cdc42 may be required for the formation of a 'sense organ' during dorsal closure, analogous to the growth cone seen in neuritogenesis, as well as being required for the dorsalward sweep of the epithelial fronts towards the midline (Jacinto et al., 2000).

The second and third classes of genes are those involved in the Drac1/JNK and TGF-β/Dcdec42 pathways respectively, and it has been proposed that traffic through these pathways may mediate a novel mechanism for cytoskeletal regulation (Ricos et al., 1999). The production of the acto-myosin cytoskeleton and phosphotyrosine-rich focal complexes at the leading edge is mediated by Drac1's stimulation of the JNK cascade, which results in the transcription of the secreted TGF-β-like molecule decapentaplegic (dpp). Embryos mutant for steps in the JNK pathway, such as hemipterous (hep) and basket (bsk), do not undergo dorsal closure, as their leading edge cells do not elongate - a phenotype which in bsk can be rescued by ectopic dpp expression (Knust, 1997). hep mutant embryos fail to extend filopodia
across the dorsal divide, and it has been suggested that the JNK pathway also regulates filopodial extension (Jacinto et al., 2000).

The secreted dpp protein binds to TGF-β receptors on neighbouring cells, namely those encoded by *thick-veins* (*tkv*) and *punt* (*put*). This results in the activation of Dcdc42, which in turn leads to the production and/or localisation of DPAK at the leading edge, accompanied by an increased production in segment border cells (Ricos et al., 1999). This may be a negative regulatory procedure - DPAK induces the dissolution of the leading edge cytoskeleton in segment border cells, and this transient relief of tension during leading edge migration might limit the bunching and shearing of the epidermis seen when dpp or Dcdc42 signalling is impaired (Harden et al., 1999; Ricos et al., 1999). This evidence has also led to the hypothesis that DRhoA and Dcdc42 might antagonise each other in competiting to regulate the cytoskeleton in leading edge cells, especially around the segment borders (Ricos et al., 1999). It is also interesting to note that in culture, there is no apparent requirement for nuclear signalling or novel protein synthesis for the induction of cytoskeletal-based cell shape changes, with factors being recruiting from existing pools. Conversely, in an *in vivo* situation, both the cytoplasmic and nuclear functions of the small GTPases are required and it has been postulated that this results from a greater demand on cellular components as morphogenesis proceeds (Ricos et al., 1999).

1.1.6.1.3 Maternal and zygotic Rhol phenotypes differ in Drosophila embryos

*Drosophila* embryos harbouring endogenous null *DRhol* alleles are similarly, if not more severely affected than those injected with the live null protein (Harden et al., 1999; Magie et al., 1999). P element insertions within the *Rhol* gene are lethal when homozygous, with disrupted dorsal closure and a failure of head to involute. Embryos generated lacking both the maternal and zygotic *Rhol* contribution are also inviable and die with segmentation defects and cuticular holes not resulting from impaired dorsal closure. Close examination of these embryos reveals that the initial patterns of *wingless* and *engrailed* expression are established correctly, but fail to be maintained. Since Wingless is a secreted molecule involved in both paracrine and autocrine signalling the maternal segmentation phenotype might result from an actin-based defect in the cells' secretory or uptake abilities, or from a disruption in a Rho-mediated signal transduction pathway, whereas the zygotic phenotype is thought to result from direct effects on the actin cytoskeleton.
1. Introduction

1.1.6.2 The generation of epithelial planar polarity requires Rho family function

The ability of epithelial tissues to form ordered, functioning tissues relies upon the ability to generate polarity, at a number of different levels. Firstly, the cells themselves must exhibit a differentiation between their apical and basal ends, dependent on the localised distribution of subcellular components, in order to allow directional processes such as vesicular transport or secretion to occur. There may also be polarity within the plane of an epithelium, for example a left-right gradient permitting cilia to beat in a uniform direction. Apical-basal polarity depends somewhat upon rearrangements of the actin and microtubule networks and can be determined by direct interaction with the local environment, such as the binding of integrins in an underlying basal lamina. On the other hand, planar polarity requires rearrangements to be made with respect to the plane of the epithelium and relies upon long-range, tissue-specific signals acting in a proximal-distal fashion since there are generally no obvious differences between the cells themselves. The reception of the signal seems to share several factors in divergent contexts and is likely to rely upon a conserved mechanism, as discussed further below.

1.1.6.2.1 Drosophila epithelial polarity

In the adult fly, all structures are derived from epithelial imaginal discs which are renowned for their planar polarisation. It could be said that the most well-characterised examples are in the wing and the eye, both of which are structures which require tightly-controlled signalling mechanisms to develop correctly. In the wing, individual cells orient themselves more or less independently in order to generate a localised actin protrusion at their distal vertices, which will become differentiated into an array of distally-pointing sensory hairs in the mature wing (Mlodzik, 1999). However in the developing ommatidia of the eye, the situation is more complex, involving the coordinated movement of whole groups of cells. The ommatidia begin life as pre-clusters of five, immature photoreceptor cells. These undergo a series of rearrangements and specifications resulting in groups of cells displaying chirality within clusters and a plane of symmetry with respect to the dorso-ventral axis of the eye imaginal disc (Mlodzik, 1999).

The Rho family of GTPases has been implicated in the generation of such epithelial planar polarity, and not merely on account of their effects on the actin cytoskeleton. RhoA mutant clones fail to proliferate in imaginal disc tissue, implying that GTPases may be required for cell growth and/or viability (Strutt et al., 1997). Clones containing hypomorphic alleles develop obvious defects - in the wing, the
hairs display abnormal polarity and there is the instance of multiple wing hairs being generated from individual cells. In the eye, planar polarity defects are also seen, such that the ommatidia are often incorrectly rotated with respect to the equator and may fail to generate R3/R4 chirality correctly (Strutt et al., 1997). Similar disruptions to planar polarity were found to result from the eye-specific expression of constitutively active Rho, or constitutively active and dominant negative Rac (Fanto et al., 2000). There is also thought to be a requirement for the proper regulation of Rhol during later eye development, as its overexpression causes the loss of certain pigment cell types and abnormal morphology in other cell types such as the rhabdomeres (Hariharan et al., 1995). It is not clear though whether these later phenotypes result from direct effects on the cytoskeleton, or Rho-regulated signalling pathways, as discussed in the next paragraphs.

Numerous genes have been identified in Drosophila which affect the generation of tissue polarity. Many of these, such as frizzled (fz) and dishevelled (dsh) (Adler, 1992; Theisen et al., 1994) are required in all tissue types, whereas others have a more restricted action, such as fuzzy, interned and multiple wing hairs (Adler et al., 1994), which are required specifically for wing hair polarity. Of these genes, it is about the function of fz and dsh that the most is known. Fz proteins have been identified as receptors for Drosophila Wingless (Wg) and its vertebrate homologues, the Wnts. Dsh is a cytoplasmic protein which is required downstream of Fz to mediate its signalling, although how this is brought about still remains to be clarified. From thereonin, the 'traditional' Wg/Wnt-mediated signals are passed on through the GSK3/β-catenin/TCF pathway, activating the transcription of target genes in the nucleus (Mlodzik, 1999). However, none of the components in this pathway generate polarity defects when mutated and so a non-canonical pathway has been proposed, in which Fz signalling acts through RhoA-mediated activation of the JNK pathway to stimulate the transcription of tissue polarity effectors. In addition, upregulation of Delta expression has been seen to be brought about by Rho and Rac, suggesting that they may provide the connection between Fz and the nucleus (Fanto et al., 2000).

There is a good deal of evidence that there may well be the existence of a second, divergent pathway. Both RhoA and the yeast STE20-related kinase Misshapen are required for pan-tissue polarity generation (Paricio et al., 1999; Strutt et al., 1997) and Dishevelled has been shown to be a strong activator of JNK cascades (Boutros et al., 1998). The two Wg/Wnt pathways are thought to diverge at the level of Dsh: Dsh has three distinct domains, an N-terminal DIX domain, similar to that found in the mouse axin gene; a PDZ domain which may mediate protein-
protein interaction; and a C-terminal DEP domain (Boutros & Mlodzik, 1999). The two N-terminal domains are thought to be required for canonical Wg/Wnt signalling, whereas the PDZ and DEP regions are needed for JNK activation in the planar polarity pathway via RhoA and Rac1 (Boutros et al., 1998). Although the majority of work carried out in this area relates to the involvement of polarity generation in Drosophila, the data shown in Chapter 4 points to a role in for RhoA-mediated activities in mediating the non-canonical Wnt pathway in vertebrates and this remains a current area of investigation in zebrafish development.

1.6.2.2 Hair cell morphogenesis

Hair cells are mechanosensory organelles found in sensory epithelia such as that lining the vertebrate ear. They are responsible for converting a mechanical stimulus such as sound or acceleration into an electrical signal by the deflection of the hair bundle - a single, tubulin-based kinocilium thronged by around a hundred stereocilia, composed of densely-packed actin filaments. The size, shape and polarity of the hair bundle are precisely controlled parameters, differing drastically between species, between different organs in the same species, and even between different locations within the same epithelium. The Rho GTPases are thought to play a role in hair cell generation - raised levels of Cdc42 are found in the sensory epithelium of the chick cochlea after trauma, suggesting a role for this protein in hair-cell regeneration and possibly involving its ability to stimulate filopodia formation (Gong et al., 1996). Mutations in the human form of diaphanous, which contributes to Rho-mediated F-actin polymerisation and organisation, lead to dominant hereditary deafness caused by the truncated version of the protein which is present (Lynch et al., 1997).

Kollmar (1999) proposed a model by which the small GTPases regulate hair-bundle formation: Rac and Cdc42 induce the polymerisation of actin at the apical surfaces of hair cells via their interactions with WASP-like and Arp2/3 proteins and the Fz pathway then signals through Rac to specify the polarity of the bundle. Cdc42 mediates the continued lengthening of the actin filaments, which are anchored to the membrane by ERM proteins. The process is completed when Cdc42 is deactivated, or the supply of actin monomers exhausted.
1. Introduction

1.1.6.3 *Rho family proteins are required during axonogenesis and dendritic branching*

The formation of the nervous system requires the precise outgrowth and targeting of neurites, the group encompassing axons and dendrites. Even in invertebrates such as *Drosophila* and *C. elegans*, the correct modulation of small GTPase function has been seen to be essential.

1.1.6.3.1 *Neuritogenesis and the growth cone*

The pathfinding ability of neurites, both in cultured cells and whole organisms, depends on the translation of extracellular cues in the environment into dynamic movements at the tip of the extending processes of the growth cone. The reading of this molecular blueprint is brought about by the growth cone, a specialised, bulbous structure with an actin-filled leading edge. Filopodia are extended forward from this leading edge, to explore the surrounding area for attractant and repulsive cues - they are quickly retracted if they encounter something they do not agree with. In contrast, any filopodia encountering positive cues are stabilised by thickening of the actin filaments within them and the extrusion of web-like lamellipodia between them. A final level of stability is generated by the incorporation of microtubules into the structure. The growth cone continues to advance, new filopodia form, and the neuronal process extends into new territory (O'Connor et al., 1990). The transduction of signals by the growth cone is not only qualitative, in terms of permissive/non-permissive environments for growth, but is also quantitative, such that a neurite will pursue the stronger of two signals (O'Connor et al., 1990).

These observations can be explained in terms of a GTPase-based model as mentioned previously. Local, transient activation of Cdc42 causes a filopodium to form, which is subsequently enhanced by positive signals from the environment, causing further actin polymerisation and focal complex formation, thus stabilising the outgrowth. This prolonged Cdc42 activation acts on Rac, causing it to stimulate lamellipodia formation - and so the process continues (Mackay et al., 1995). This importance of focal complexes to this process has been seen in PC-12 lines generated to lack most of their vinculin, a major constituent of focal complexes. Although these cells could extend filopodia of the same length and at the same rate as wild-type cells, they were shorter-lived, and there was a destabilisation and premature retraction of the lamellipodia (Varnum-Finney & Reichardt, 1994).
Tyrosine kinases may also be involved in the regulation of filopodial extension, either by acting on actin filament assembly, or by regulating the association between cell-substratum adhesions and retrograde actin flow at the leading edge of the growth cone (Mackay et al., 1995). Indeed, the speed of this retrograde actin flow is also thought to be involved in regulating axon outgrowth - in cultured Aplysia neurons, the rate at which the growth cone advances is seen to be inversely proportional to the speed of retrograde actin flow (Lin & Forscher, 1995). This actin-cycling is regulated at some level by myosin, as it can be inhibited by myosin-blocking drugs or mutant proteins (Lin et al., 1996).

Exactly how the activation of the GTPases by extracellular signals is translated into downstream reorganisation of the actin cytoskeleton still remains relatively oblique. A RhoGEF has been identified from a yeast two-hybrid screen which is specific for RhoA, the overexpression of which in N1E-115 cells mimics the neurite collapse and cell rounding seen when V14Rho is introduced in these cells (Gebbink et al., 1997). The same screen also led to the identification of p116Rip, a novel Rho-interacting protein with apparently opposite effects: overexpression mimicked treatment with N19Rho or C3, inducing neurite extension (Gebbink et al., 1997). No doubt future work will lead to the description of further examples of such factors, and the mechanisms underlying neuronal remodelling will become clearer.

1.1.6.3.2 Rho GTPases are required for dendritic morphology

Although neuronal cell lines have provided evidence as to the roles of the Rho family in neural modelling, perhaps a more vivid elucidation of their function comes from investigations using primary cultured neurons and, even more pertinently, in vivo assays. It may be the case that cell lines do not always faithfully reproduce the events occurring in 'real-life' - for instance, in embryonic chick dorsal root ganglion cells (DRGs), C3 is found to stimulate axon outgrowth but produces abnormal growth cones lacking filopodia and lamellipodia (Jin & Strittmatter, 1997), whereas they were present in growth cones induced by C3 in N1E-115 cells (Kozma et al., 1997).

A key feature of neuronal differentiation in the central nervous system in any animal is the acquisition of cell type-specific morphologies. The dendrites are the sites where neurons receive, process and integrate signals from presynaptic partners and these features are primarily dictated by the morphology of the dendritic tree. The arrangement of a neuron's dendrites, for example, determine which cells it can interact with and what kind of interactions it is capable of. In higher vertebrates,
developing cortical neurons undergo extensive dendritic growth, branching and remodelling to achieve their pyramidal and non-pyramidal morphologies. This plasticity is heavily reliant upon the actin cytoskeleton, and is regulated by RhoA, Rac1 and Cdc42 in cultured cortical neurons (Threadgill et al., 1997). Transfection of dominant negative forms of all three GTPases, or C3 exoenzyme cause a reduction in the number of primary dendrites in the multipolar, nonpyramidal neurons, and a decrease in the number of basal dendrites in pyramidal cell-types. Constitutively active GTPases increase the numbers of each accordingly. Additionally, dominant negative Rac and Cdc42 both inhibit the morphological shift from pyramidal to nonpyramidal which occurs over time both in vivo and in vitro - thus, the GTPases are likely to play an important role in the shaping of neurons during cortical development.

An higher level of dendritic specialisation occurs in certain mammalian neuron types, including the cortical and hippocampal pyramidal neurons and the cerebellar Purkinje cells (Rall, 1964). The dendritic branches are further differentiated into spines, which are the primary sites for excitatory synapse integration, and there is evidence to suggest that even in the adult, dendritic spine morphology is still being actively altered in response to such factors as synaptic activity and expression (Nakayama et al., 2000). Using the fairly novel technique of biolistic transfection, Nakayama et al. (2000) found that both RhoA and Rac1 are required for the maintenance of dendritic spines in rat hippocampal slice cultures - dominant negative Rac1 caused a progressive loss of spines, whereas constitutive activation of RhoA caused Rho-kinase dependent simplification of dendritic branching. Furthermore, constitutively active Rac was found to have little effect on the branching pattern of the dendrites, but it did bring about a loss of normal spines, there being instead an array of numerous, overlapping filopodia-like structures and membrane ruffles. Likewise, similar results were found in an in vivo assay for Rac's role in the Purkinje cells of the cerebellum. Transgenic mice were made expressing constitutively active Rac1 under a Purkinje-specific promoter. The growth and the branching of the cells were little different from wild-type; however, the dendritic spines were severely affected, being smaller and more numerous with the occasional supernumerary synapse being present. The mice themselves were ataxic, but otherwise unaffected (Luo et al., 1996). These data suggest that although Rac functioning is required for a normal dendritic branching pattern, the spines are more sensitive to over-stimulation of Rac than the branches are.
1.6.3.3 *A neural specific Rac induces neuritogenesis in the chick*

Cloning and subsequent *in situ* analysis revealed the presence of several ubiquitously expressed Rho family members in the chick, as mentioned earlier. However, quite striking was the existence of a Rac1 isoform, termed cRaclb, the expression of which appeared to be restricted to the developing nervous system (Malosio et al., 1997). Over-expression of cRaclb in cultured embryonic retinal neurons induced a dramatic increase in neurite outgrowth and branching; as might be expected, a dominant negative form blocked the formation of any such processes. However, it was also found that the introduction of cRacla in either conformation had little effect on neuronal morphology, whereas both cRacla and cRaclb could induce actin-rich membrane protrusions in embryonic fibroblasts (Albertinazzi et al., 1998). These results raise interesting issues regarding the specificity of GTPase activity within specific cell types - even though cRacla is expressed in retinal cells, it seems to have no effect on the modelling of neuronal morphology.

1.6.3.4 *Upstream signals are required for GTPase activation*

The developing embryonic nervous system presents a complex environment, through which the growth cone must navigate. It is known that the detection of guidance cues, perhaps by the binding to a receptor of a growth factor or ligand, is necessary for the progress and appropriate targeting of the developing neuron (Gallo & Letoumeau, 1998). A considerable amount is known about the effect of such cues on axon guidance - for instance, the expression of the Ephrin ligands in the developing brain is known to act as a series of gradients, the reading of which by Eph receptors permits the targeting of retinal ganglion axons to the correct areas of the tectum (Brennan et al., 1997; Frisen et al., 1998; Nakamoto et al., 1996). *In vivo* experiments in which *Xenopus* retinal ganglion cells were transfected with different GTPase constructs showed that the Rho family are required for pathfinding in the tectum - axon extension, target selection and target recognition were all compromised by the expression of dominant negative RhoA and the over-expression of wild-type Cdc42 (Ruchhoeft et al., 1999), suggesting that regulation of the certain aspects of the GTPase-mediated pathways is necessary for the ability to read and act on guidance cues. It is also noteworthy that constitutively active forms of Rho, Rac1 or Cdc42 completely inhibited axon extension and growth cone formation (Ruchhoeft et al., 1999), which conflicts somewhat with data achieved in other systems - one might expect neurite overgrowth in response to constitutively active Rac, but this is not the case here.

48
The mechanism by which these upstream signals are translated by the Rho family into actomyosin-based movements is less well characterised. Ephrin-A5 is known to induce growth cone collapse in cultured retinal ganglion cells (Drescher et al., 1995; Monschau et al., 1997). The role of Rho in this process has been investigated, and it is found that in cultured embryonic retinal ganglion cells, ephrin-A5 brings about a dramatic increase in the amount of activated, GTP-bound Rho and a downregulation of activated Rac. These effects are likely to underlie ephrin-A5-mediated growth cone collapse - ADP-ribosylation of Rho with C3 or the inhibition of Rho-kinase with Y-27632 in these cells considerably reduced the level of collapse induced by the ligand, implying a key role for the Rho family in the reception of repulsive cues during the development of the nervous system (Wahl et al., 2000).

The neurotrophin receptor, p75NTR, is a membrane glycoprotein expressed in many cell types in the developing nervous system. In many cases, it is associated with cells that are having to project axons over long distances, or are actively modelling their dendrites (Yamashita et al., 1999). Again in a yeast two-hybrid system, p75NTR was found to interact with Rho and in vitro data suggest that whilst p75NTR acts as a constitutive activator of Rho, this activation can be downregulated by the binding to it of neurotrophins such as nerve growth factor, and this can lead to neurite proliferation in cultured neurons. It is interesting to note that p75NTR mutant mice lacking the neurotrophin-binding domain of the receptor have reduced outgrowth of sensory and motor axons, which may well result from the over-activation of Rho affecting neurite outgrowth in an in vivo situation (Yamashita et al., 1999). Migrating neural crest cells are known to express p75NTR and, in the chick, RhoB (Liu & Jessell, 1998), so the regulation of Rho function by neurotrophic factors may not be restricted to neurons but may also function in the correct streaming of neural crest cells.

1.6.3.5 RhoA is required for nervous system development in Drosophila

There is a putative role for RhoA in the modelling of the Drosophila central nervous system. Mosaic larvae were generated with homozygous null RhoA clones in the mushroom body neurons of the developing brain (Lee et al., 2000). In wild-type larvae, a mushroom body neuroblast clone examined at the third instar stage contains over 150 neurons, rising to over 500 neurons in the adult which project to 5 different lobes. However in the mutant clones, this number decreases to and remains at 10-12 cells, projecting only to a single, medial lobe. The axonal morphology of this depleted group of cells remains phenotypically wild-type; there is though evidence of dendritic over-extension. Hence, RhoA is thought to be required at two
critical stages in development of the *Drosophila* nervous system: firstly, it regulates cytokinesis, such that a knock-out causes a cell division arrest with the occasional presence of multi-nucleate cells; and secondly, it is required to limit dendritic growth during early neuronal differentiation. This may reflect a common mechanism for dendritic modelling and synapse formation which has been conserved during the course of evolution, both in invertebrates and vertebrates.

1. Introduction

1.1.6.3.6 *Drac1* is required for sensory and motor neuron guidance in *Drosophila*

The initial cloning and expression of the *Drosophila* homologues of Rac1 and Cdc42, *Drac1* and *Dcdc42*, revealed them to be widely expressed during embryogenesis with a particular enrichment in the developing nervous system and mesoderm (Luo et al., 1994). Using enhancer-trap lines, neural-specific expression of V12Rac caused a loss of axon initiation and elongation depending on the time of onset of expression, with an abnormal actin distribution within affected cells. An attenuated version of this phenotype was seen with dominant negative Rac1 expression. When the same experiments were performed using Cdc42 constructs, a qualitatively different phenotype was seen, with constitutive activation having a more general disruptive effect on neuronal positioning and dendrite formation as well as sensory axon outgrowth (Luo et al., 1994). The effects seen imply that in *Drosophila*, there is little redundancy of function between Rac and Cdc42 as seen by the severe, embryonic lethal phenotypes produced by over-activation of either. It might also be implied that there is a hierarchical GTPase activation in this system, in which the constant activation of Cdc42 has a knock-on effect on downstream events mediated by Rac1.

Using *Drosophila* intersegmental nerve b (ISNb) motor axon growth cones as a model system, more on this topic has been elucidated (Kaufmann et al., 1998). Expression of constitutively active Drac1 or Dcdc42 causes growth cone arrest, with an identical phenotype for both proteins. However, it is examination of the dominant negative phenotypes that provides a better insight into the events occurring: Cdc42 incurs only a subtle defect in that a small proportion of ISNb axons fail to reach their final destinations on account of growth cone arrest. In contrast to this, dominant negative Rac1 has no effect on the progression of axon outgrowth but there are severe defects in axon guidance (Kaufmann et al., 1998). Hence, there is support here for the hypothesis that the different GTPases regulate different aspects of neuritogenesis during nervous system development, in that Rac is required for the reception of guidance cues, whereas Cdc42 is needed for the progression and maintenance of the growth cone. Comparing these results with those from Luo et al.
(1994), it may also be suggested that in different cell types, the GTPases can play context-dependent roles.

1.1.6.3.7 The role of other GTPase family members in the nervous system

Although Rho, Rac and Cdc42 are the most familiar and well-examined of the Rho family, it has come to light that other, more obscure family members may also play important roles during the patterning and development of the nervous system. Firstly, a role has been shown both in vitro and in vivo for TC10. In the rat brain, TC10 expression levels are low in the foetus, but gradually increase with age - even in the adult though, levels are not as high or the expression pattern as widespread as for Rho, Rac or Cdc42 (Olenik et al., 1997; Tanabe et al., 2000). However, a role for TC10 is implicated in nerve regeneration - after motor neuron axotomy, expression of TC10 is upregulated in the injured tissue to a greater extent than Rho, Rac or Cdc42, suggesting that it might be required for neurite outgrowth in response to injury. Indeed, in cultured rat dorsal root ganglion cells (which induce TC10 expression after transection in vivo), expression of constitutively active TC10 is found to promote neurite extension in the absence of other factors (Tanabe et al., 2000). Thus, TC10 may play a role in mediating axon outgrowth, perhaps in a pathway which responds specifically to injury - however, it is yet clear whether it functions independently, or via crosstalk with other GTPases. The inactivation of Rho signalling is found to promote CNS regeneration (Lehmann et al., 1999), and so both Rho and TC10 may represent possible therapeutic targets for neural injury.

In the nematode C. elegans, a widely-expressed Rac1 homologue has been identified (Chen et al., 1993). However, it is the Cdc42-related gene Mig-2 about which more is known. Alleles of Mig-2 had been identified in several genetic screens for mutants with behavioural phenotypes or altered cell migration and positional cloning of this gene identified it as being a Rho family member. Analysis of mutant animals reveals different phenotypes depending on the activation status of the protein, such that persistent activation affects the migration of certain cell types, including a subset of neuronal cells. Moreover, a striking effect is seen on the hermaphrodite-specific neuron (HSN) which is truncated in a small percentage of animals and is aberrant in its guidance in a larger proportion of others, failing to project axons to the ventral nerve cord. Loss of function Mig-2 alleles have less severe effects, slowing the rates at which a subset of Q lineage cells migrate (Zipkin et al., 1997). These results suggest that although the functioning and regulation of Mig-2 is required for proper cell migration and axon outgrowth in C. elegans, there
is a considerable degree of redundancy occurring in most cells as only a fraction of those in the embryo are affected.

1.1.6.4 RhoB and neural crest formation

Whereas many of the Rho GTPases so far examined appear to be widely expressed during embryonic development, a striking exception has been identified in the chick, with the expression pattern of \textit{RhoB} (Liu & Jessell, 1998). \textit{In situ} hybridisation revealed it to be expressed exclusively in the dorsal neural tube and early in migrating neural crest cells during embryonic development. Further investigation revealed an essential role for RhoB in the delamination of neural crest cells from the neural tube - however, this requirement would appear to be transient and restricted to a narrow timepoint of development, as ablation of RhoB expression did not prevent the specification of the neural crest, nor its later migration, which are the stages temporally flanking the cell delamination (Liu & Jessell, 1998). Thus, Rho GTPases can act during specific programmes of development and can be induced as a response to signalling molecules, the instance here being BMP4.

1.1.6.5 Rho GTPases and muscle development

Alongside the roles for Rac and Cdc42 in axon guidance in \textit{Drosophila}, they have also been found to be required in myoblast fusion during muscle development. Expression of constitutively active Drac1 in mesodermal cells was found to block myoblast fusion, inhibiting the formation of multinucleate syncitia, but did not block affect general muscle patterning, or the expression of muscle-specific genes. Conversely, the dominant negative form delayed and subsequently over-stimulated myoblast fusion, giving rise to thinner, inappropriately-shaped muscle fibres. The ectopic expression of mutant Cdc42 constructs in the mesoderm gave less severe phenotypes, with the constitutively active form giving a mild muscle patterning problem without affecting myoblast fusion and the dominant negative only giving subtle defects in certain segments (Luo et al., 1994). This suggests that in \textit{Drosophila}, both Rac and Cdc42 are required for muscle specification but they are likely to act in parallel pathways, as judged by the effects mutant forms have on development.

In addition to the effects seen on the cytoskeleton, the functioning of RhoA in cell differentiation has been shown in cultured mouse fibroblasts. The induction of \textit{MyoD} expression is controlled by a serum response factor (SRF) mediated signalling cascade. Rho, Rac and Cdc42 have been shown to mediate transcriptional activation
response to serum or lipophosphatidic acid by means of a novel signalling pathway. It is this cascade that is thought to be required for MyoD induction, as transfection of the myoblasts with dominant negative human RhoA, or culturing in C3 exoenzyme leads to a marked decrease in the levels of MyoD protein present without affecting a similar marker of muscle differentiation, Myf5 (Carnac et al, 1998).

1.1.7 Clinical perspectives

1.1.7.1 Rho GTPases and the immune system

1.1.7.1.1 Rac mediates neutrophilic responses

As mentioned earlier, Rac is known to regulate the oxidative burst, leading to the production of reactive oxygen species, which act in the immune response as a toxic agent for the destruction of pathogens. This system is vital for the correct functioning of neutrophils, the predominant phagocyte species protecting against bacterial and fungal infections. Inflammatory signals result in a rapid response from these cells, causing them to migrate across the endothelial lining of the blood vessel to the site of infection. They are responsible for ingesting the foreign microbes and destroying them via a combination of oxygen-dependent and oxygen-independent mechanisms. The haemospecific Rac2 is known to be critical for these processes, being involved both in the remodelling of the actin cytoskeleton to coordinate the chemotactic migratory response; and in the regulation of the oxidative burst mediated by NADPH. A dominant negative mutation at position 57 in the Rac2 protein, converting the conserved Asp into Asn, results in neutrophils which do not form lamellipodia and cannot migrate, as well as being unable to mediate superoxide production. The consequences of this are a severe immunodeficiency syndrome (Ambruso et al., 2000).

Other roles for GTPases have been implicated in the regulation of immune system development. Severe congenital neutropenia (SCN) is a myelopoiesis disorder, where cells are found to arrest at the promyelocyte or myelocyte stages of development, leading to a severe decrease in the number of circulating neutrophils. Patients given granulocyte colony-stimulating factor to boost their neutrophil levels are found to have cells with an overexpression of RhoGDI but a reduction in absolute Rac2 protein levels, implying a role for both of these proteins in normal neutrophil development (Kasper et al., 2000).
GTPases may have a more general function in haemopoietic lineages. As discussed earlier, patients suffering from Wiskott-Aldrich syndrome have an immune deficiency and an increased risk of lymphoid malignancy - their white blood cells have an abnormal morphology with decreased motility and a lack of processes such as filopodia, resulting in an inability to 'catch' antigens and giving rise to a poor immune response (Thrasher & Kinnon, 2000). The mutant protein responsible for this illness, WASP, is known to be a Cdc42 downstream effector, with patients lacking the haemo-specific form (Millard & Machesky, 2001). The exchange factor, Vav, is also thought to function in haemopoiesis - mouse embryonic stem cells with a homozygous null Vav gene produce reduced numbers of B and T cells, with the T cells being deficient with respect to T-cell receptor signalling and the positive selection of mature cells (Fischer et al., 1995). There is also a requirement for Vav in early embryonic development - embryos generated from homozygous null clones do not form the trophoblast lineage correctly and fail to implant (Zmuidzinas et al., 1995).

1.1.7.1.2 A role for Rho in the thymus

The thymus gland is the seat of T cell production. However, more than 95% of the cells developing there die immaturely - there is a system of positive selection in the thymus, by which only cells capable of recognising foreign peptides presented in the context of self molecules are selected for survival (von Boehmer, 1992). A role for Rho in the thymus has been shown by the data that transgenic mice generated with thymus-specific C3-exoenzyme expression display smaller thy mi, with decreased cellularity within them and a severe disruption of morphology (similar effects were seen in the spleen, suggesting a mechanism by which the thymus regulates splenic differentiation). Although the T cells are found to mature normally in such animals, they are present in decreased numbers in the peripheral circulation and a close examination of the immature cells in the thymus shows many of them to be locked in the G\textsubscript{1} phase of the cell cycle. Consequently, blocking Rho does not affect thymocyte differentiation but is found to block proliferation by affecting progression through mitosis (Henning et al., 1997).

Rho is also thought to control the p53-dependent cell-survival checkpoint during the development of pre-T cells - a loss of Rho function results in apoptosis of these cells which can be overcome by an accompanying p53 knock-out. A later, p53-independent thymocyte selection point is also regulated by Rho - in this case, the apoptosis caused by the loss of Rho can be relieved by the expression of Bcl-2.
(Costello et al., 2000). It is not clear though how Rho regulates either of these processes.

1.1.7.2 Rho GTPases and the circulatory system

1.1.7.2.1 Rho is involved in angiogenesis

Angiogenesis is the process by which capillaries are formed from pre-existing blood vessels and thus plays an essential role in embryogenesis and wound healing; the flip-side of this is that angiogenesis is crucial to the growth and sustenance of certain tumours and is therefore a prime target for anti-cancer therapeutics. The process involves the degradation of basement membranes, the locomotion of endothelial cells and their subsequent proliferation. It is found that the formation of capillaries by human umbilical vein endothelial cells (HUVECs) in vitro and by chorioallantoic membrane in the living chick embryo can be inhibited by C3 and the Rho-kinase blocking agent, Y27632, suggesting a key role for Rho in capillary formation. It is of clinical interest that oral dosing with Y27632 can block artificially induced angiogenesis in mouse skin in vivo, and this may be an avenue to be pursued with respect to drug development (Uchida et al., 2000).

1.1.7.2.2 Alteration of Rho expression leads to heart failure

Cardiac myocyte hypertrophy is an adaptive response leading to increased myocardial contractility and ultimately, in severe cases, heart failure. Transgenic mice were generated that postnatally expressed wild-type or constitutively active forms of the Rho GTPase in a cardio-specific manner. Whereas the overexpression of Ras leads to cell growth and hypertrophy, that of Rho leads to lethal heart failure with the upregulation of many genes known to be markers of hypertrophy. Even the heterozygous pups are affected to varying degrees, displaying an increased incidence of premature death. In vivo experiments with cardiac myocytes showed that RhoA can be upregulated by angiotensin-II; and endothelin-1-induced hypertrophy can be blocked by the Y-27632 Rho-kinase blocking agent (Clerk & Sugden, 2000). Investigations in cultured myocytes have also suggested a link between the activation of the purinergic receptors P2X and P2Y and the activation of RhoA and its downstream pathways. The activation of P2X and P2Y is known to regulate vasodilation, vasoconstriction and vascular smooth muscle proliferation in vivo, thus these processes may also be partially dependent on Rho signalling (Sauzeau et al., 2000).
Rac1 may also be involved in certain forms of cardiomyopathy. Transgenic mice expressing activated Rac1 in the myocardium displayed two, distinct phenotypes: a dilated phenotype resulting in death shortly after birth; or the less severe transient hypertrophy seen in juveniles which resolved with age. The cardiac myofibrils appeared normal in both cases but PAK was translocated from the cytosol to the cytoskeleton, suggesting that constitutive activation of Rac-mediated signalling pathways may be the underlying cause of the cardiac phenotypes seen (Sussman et al., 2000).

Although the pathways leading to hypertrophy mediated by the Rho GTPases are still not well understood, models such as these imply that they play an important role in the progression of hypertensive heart disease and again, may one day play a role in the development of therapeutic agents for its treatment.

1.1.7.2.3 Rac contributes to post-ischaemic reperfusion injury

Reperfusion following oxygen starvation can prove to be highly detrimental to tissues in vivo, with necrosis and apoptosis often being the outcome of ischaemia in organ transplantation, strokes, or myocardial infarction. Reperfusion contributes to tissue injury by the generation of reactive oxygen species (ROS), which initiate a series of events leading to inflammation and oxidative damage to lipids, proteins and nucleic acids. As Rac1 is known to mediate the production of ROS via its activation of the NADPH oxidase complex in phagocytic and non-phagocytic cells, an in vivo mouse model of hepatic ischaemia/reperfusion injury was developed in which dominant negative N17Rac was delivered to the injured site. The presence of N17Rac was found to block superoxide and hydrogen peroxide generation by the reperfusion-induced response, and also decreased the level of oxidative damage to lipids. An additional level of injury is mediated by the induction of inflammatory response genes by a NF-κB mediated transcriptional cascade, which is sensitive to the redox state of the cell - this NF-κB signal is also blocked in the presence of N17Rac. However, neither necrosis nor apoptosis is completely abrogated by the inhibition of Rac, implying that there are alternative mechanisms for ROS generation, such as xanthine oxidase or the mitochondrial electron transport chain (Ozaki et al., 2000).
1.1.7.3 Rho GTPases and cancer

1.1.7.3.1 Rho family members may be implicated in oncogenic transformation

Although the Ras family of small GTPases have been widely implicated in tumour formation and progression. However, with the possible exception of RhoC (see 1.1.7.3.3), there is little evidence that the Rhos themselves are oncogenic, whereas interacting factors such as the Rac GEF Tiam-1 are more widely implicated as proto-oncogenes (Werner & Manseau, 1997). That is not to say that Rho, Rac and Cdc42 themselves may not play important roles. NIH-3T3 cells transfected with wild-type or V12Rho show a reduced dependency on serum for growth and have reduced anchorage and can induce tumour formation in nude mice (Fort, 1999). Similar effects are seen with Rac and Cdc42, with Rac being able to reduce cell contact inhibition. It may well be that Rho pathways are activated downstream of Ras-controlled pathways (Qiu et al., 1995) -Ras-induced membrane ruffling in cultured cells requires functioning Rac (Ridley et al., 1992), and in the fission yeast S. pombe, signals regulating morphology are mediated by Ras through Cdc42 (Chang et al., 1994).

There may be an essential role for Rho in the repression of transformation, particular in specific tissues in vivo. Transgenic mice expressing C3 under the control of a thymocyte-specific promoter develop aggressive malignant lymphomas, showing that an abrogation of Rho function is associated with a predisposition to lymphoid cell transformation - however, the cancer seen does not have an immediate onset, so there may be additional, redundant mechanisms earlier in development which protect against the Rho knockout (Cleverley et al., 2000).

Nevertheless, there still remains debate about the exact role the Rho family might play, if any, in transformation and whether such effects would be exerted through effects on the cytoskeleton or downstream signalling cascades, or perhaps a combination of the two.

1.1.7.3.2 Rho and hamartoma formation

Patients with the syndrome Tuberous Sclerosis are prone to the formation of tumorous growths known as hamartomas. The tumour suppressor gene TSC1 makes the protein hamartin, the function of which is largely unknown. However, over-expression of hamartin results in the activation of Rho, inducing the formation of stress fibres and focal adhesions. The interaction of hamartin with ERM proteins is
1. Introduction

said to be required for the serum- or LPA-mediated activation of Rho. Conversely, an inhibition or a loss of hamartin leads to a reduction in adhesion and ultimately, the formation of hamartomas - firing down the Rho pathway may well be the rate-limiting step in tumour formation (Lamb et al., 2000).

1.1.7.3.3 RhoC may be required for metastasis

Although on the basis of their homology to Ras the Rho GTPases ought to act as oncogenes, there was little if any evidence to support this until recently. Innovations in DNA microarray technology has allowed the analysis of gene expression profiles in different cell types. Melanoma cells were selected for a highly metastatic phenotype and were found to have enhanced expression of genes which could be broadly divided into two categories: those involved in extracellular matrix assembly, and those involved with actin. Surprisingly, RhoC is one of these enhanced genes, an increase in its expression being correlated with the progression of pancreatic adenocarcinoma to metastasis. RhoC can enhance metastasis when over-expressed; conversely, dominant negative RhoC can inhibit it, as can N19RhoA. However, despite their high level of sequence homology, RhoA and RhoC have opposing effects on the migratory and invasive capacities of cells, the former blocking them, the latter enhancing. Perhaps more revealing is the fact that when expressed at comparable levels, RhoC is a far more efficient motogen than RhoA in metastatic cells - this opens up a whole new vista with respect to the regulation of Rho family members that were previously assumed to be equivalent, as well as providing possible new therapeutic targets (Clark et al., 2000).

1.1.7.4 Wound healing

1.1.7.4.1 Rho GTPases are necessary for embryonic wound healing

In recent years, the healing of wounds made in the embryo has become a paradigm for the study of tissue repair and the processes involved therein. Compared to the adult situation, wound healing in the embryo is rapid and occurs perfectly, without an inflammatory response or the production of fibrous scar tissue. In the adult, wounds heal by the contraction of the exposed connective tissue beneath the lesion, whilst simultaneously, the overlying epidermis migrates to envelop the exposed area. Although the embryo utilises a superficially similar combination of epithelial movements and connective tissue contractions, the subcellular mechanisms are rather different.
At the edge of an embryonic wound, the cells are smooth and taut, remaining attached to the underlying basal lamina as they move forward and in the front row, a thick actin cable assembles within two minutes of the lesion occurring (Grose & Martin, 1999). It is this cable which provides the driving force for wound closure - the classical comparison is to a purse-string, in which the contraction of the cable draws the edges together (Martin & Lewis, 1992). This process is known to be dependent on Rho: loading the leading edge cells with C3 exoenzyme blocks the assembly of the cable (Brock et al., 1996b), as does blocking the de novo synthesis of F-actin with cytochalasin D (McCluskey & Martin, 1995). Rac-blocking agents have no such effects.

Taken at face value, the epithelial movements of embryonic wound healing are startlingly similar to those involved in the process of dorsal closure in the *Drosophila* embryo - cells at the lateral epithelial edges migrate over and cover up the exposed, transient dorsal membrane, the amnioserosa which itself aids closure by shrinking. The dorsal sweep by the lateral epithelium is again brought about by the formation and contraction of an actin purse-string. However, there are patently differences between the two processes: firstly, in wound healing, cells gradually withdraw from the leading edge as the circumference of the wound decreases, whereas in dorsal closure, the cells all remain at the leading edge and become longer and thinner as closure proceeds (Grose & Martin, 1999). In addition, only Rho is thought to be necessary for wound healing, whereas Rho, Rac and Cdc42 are all needed for dorsal closure - Cdc42-induced filopodia formation is a key element in the process (Jacinto et al., 2000).

The 'start' and 'stop' signals regulating embryonic wound closure are as yet uncharacterised. In adult wounds, initiation could easily be triggered by platelet degranulation and the surge of growth factors and inflammatory signals at the wound site (see 1.1.7.4.2 below) - however in the embryo, such a response may not be triggered. Certainly, wound healing can occur in embryos at developmental stages prefiguring the development of the immune system, such as epiboly-stage zebrafish embryos (K. Woolley, unpublished). The intact epithelium is under tension and it has been proposed that the sudden relief of such when wounding occurs generates a mechanical shock. This may then trigger a set of stretch-sensitive cues which culminate in the formation of the contractile actin purse-string and eventual wound closure (Brock et al., 1996b). There may also be a calcium-sensitive component to this response: mechanically damaged cells at the wound's edge become transiently leaky and allow the ingress of calcium and other ions, which may go on to stimulate Ca²⁺-sensitive signal transduction pathways. Indeed, in injured *Xenopus* oocytes,
membrane healing occurs within seconds, but cytoplasmic restitution is slower and depends on an actin purse-string. The entire healing process in *Xenopus* requires Ca\(^{2+}\) ions and their influx from the surrounding fluid may be the cue for cytoskeletal organisation (Woolley & Martin, 2000).

1.1.7.4.2 Rho regulates platelet aggregation

Platelets are cell fragments which bud off from megakaryocytes in the bone marrow and circulate in the bloodstream to help mediate blood clotting in response to blood vessel damage or soluble signalling factors. A stimulus such as this results in platelet activation and the triggering of intracellular signalling pathways - a \(\beta_3\) integrin is activated in the membrane and the platelets aggregate with each other and the clotting protein fibrinogen. Thrombin is known to be a stimulus which can trigger this cascade and it brings about Rho-mediated events such as the activation of PI 3-kinase and rapid reorganisation of the cytoskeleton in soluble platelets - treatment of platelets with C3 exoenzyme inhibits thrombin-mediated aggregation (Morii et al., 1992; Zhang et al., 1993). This having been said, there does remain debate in the literature about which GTPase may be involved in this process. Hartwig et al (1995) show that thrombin-induced actin polymerisation is PIP\(_2\)- and Rac-dependent via the activation of PIP-5 kinase, with Rac and not Rho mimicking the effects of thrombin. It is likely though that both Rho and Rac are required to mediate different aspects of platelet aggregation. Additionally, there may be effects on other cell types exerted by activated platelets - those incorporated into blood clots secrete platelet-derived growth factor (PDGF), which is known to induce cell proliferation and Rac-mediated actin rearrangements resulting in membrane ruffling (Ridley et al., 1992; Ross et al., 1986). It is clear to see how PDGF secretion may aid wound healing, by inducing cells to migrate to the wound site and to proliferate once there.

Considering the following *in vitro* data though, there may be a case for different mechanisms mediating wound healing, albeit in a simplified experimental system. Wounded MDCK cell layers close by Rac-dependent cell crawling, with lamellipodia forming at the margins and the absence of an actin-purse string. Rho and Cdc42 are found not to be absolutely necessary for closure, as their inhibition leads to an increase in the irregularity of wound healing but does not prevent it from occurring. Conversely, dominant negative Rac blocks lamellipodia formation but will only prevent wound closure if injected into several rows of cells leading away from the margin's edge: injecting the first row of cells alone has no effect on closure and it is proposed that the Rac-dependent forces and Rac-mediated actin assembly
are distributed over several rows of cells at the leading edge (Fenteany et al., 2000). This conflicts with the absolute requirement for Rho in the leading edge cells of the \textit{in vivo} wound models. However, it is not known to what extent, if at all, this Rac-based movement is relevant in wound healing \textit{in vivo}, embryonic or otherwise.

Even more speculation exists as to the 'stop' signals in embryonic wound healing. When the two edges finally meet, they cease forward movement on account of 'contact inhibition' (Abercrombie, 1979) after having over-run into each other slightly, prior to actin cable disassembly (Brock et al., 1996a). Although there is no evidence to confirm or refute this assertion, it seems highly likely that stoppage is similarly mediated by Rho, by the downregulation of its intracellular effects.

1.1.8 Summary

It is clear to see the essential role the Rho GTPases play in eukaryotic cells, with their subcellular functions being coordinated into a response which may determine the precise development of entire systems. The involvement of these proteins during embryonic development is of particular interest and it will be interesting to examine the function of these proteins in vertebrate development \textit{in vivo}, using the zebrafish as a developmental paradigm.
1. Introduction

Part II

The Zebrafish as a Developmental Model

1.II.1 The rise of the zebrafish

For the past two decades, this small, freshwater tropical fish has risen in popularity to become one of the key experimental models for studying vertebrate development. The reasons for this are manifold. The fish are hardy and inexpensive to keep in considerable numbers; they reach maturity at three to four months of age, with females being capable of laying several hundred eggs once a week - external fertilisation allows the collection, observation and manipulation of the embryos. Perhaps though, one of the key factors in the zebrafish's increase in acceptance is the embryos themselves: being transparent, they can be studied under a dissecting microscope, with it being relatively simple to follow individual cells. They also develop rapidly, having all their main tissues specified by 24 hours post fertilisation (hpf) and becoming free-swimming larvae at 2-3 days of development at 28°C. Thus, the zebrafish has made the transition from the home aquarist's favourite to being an esteemed tool for the analysis of vertebrate embryonic development.

1.II.1.1 The development of the zebrafish embryo

The stages of embryonic development after fertilisation have been well-described, as shown in fig. 1.3 (Kimmel et al., 1995), with detailed fate maps showing the earliest positions of tissue progenitors (Kimmel et al., 1990; Melby et al., 1996) and later analyses describing the development of the nervous system (Strahle & Blader, 1994) and posterior structures (Kanki & Ho, 1997). The following sections briefly discuss the early cell divisions and movements leading to axis formation and commitment to specific fates.

1.II.1.1.1 The development of the blastula

The generation of animal-vegetal polarity in the zebrafish embryo is evident immediately after fertilisation - translucent cytoplasm can be seen to stream towards the animal pole, where it amasses as the blastodisc, whereas the opaque, granular yolk is segregated to the vegetal hemisphere. Cytoplasmic connections will remain between the nascent blastomeres and the yolk during the early cleavage stages. The first cell division occurs 45 minutes after fertilisation, with subsequent cleavages occurring at 15 minute intervals from thereonin (Kimmel et al., 1995).
Fig. 1.3 Camera lucida drawings of the zebrafish embryo at various embryonic stages

Arrowheads on the prim-6 embryo indicate the hatching gland (on yolk ball) and primordium of the posterior lateral line (dorsal side)

[adapted from Westerfield et al.]
1. Introduction

At the 128-cell stage, the eighth zygotic cell cycle, the embryo becomes known as a blastula, in a stage which will last until the beginning of gastrulation five or six cell cycles later. Blastula-stage embryos undergo a number of critical processes - mid-blastula transition (MBT), which marks the initiation of zygotic transcription; the formation of the yolk syncitial layer (YSL); and the beginning of epiboly (Kimmel et al., 1995). MBT begins at cell cycle 10 and marks the loss of cell cycle synchrony, or to be more precise, the loss of the metasynchronous waves of cell division that, at later stages, emanate from the animal pole and propagate towards the blastodisc margins. This loss of synchrony is accompanied by a lengthening of the cell cycle and its onset is said to be controlled by the nucleocytoplasmic ratio, such that cell cycle length varies with the reciprocal of cell volume - asynchrony is maintained by asymmetric cell division, in a model which proposes that the titration of a maternally-supplied cytoplasmic component maintains the length of the cell cycle. Hence, the end of MBT, in cycle 13, occurs when this titration is complete and the nucleocytoplasmic ratio no longer dictates cell cycle length (Kane & Kimmel, 1993).

The YSL is an organ that appears to be unique to teleost fish embryos. An extraembryonic structure, it forms during cycle 10 by the collapse and fusion of the marginal blastomeres, which continue to divide metasynchronously irrespective of MBT. The YSL lies between the yolk and the blastomeres and is suggested to play a nutritive role during embryogenesis, remaining as an intact layer contacting the inner surface of the blastodisc during the subsequent processes of epiboly and later, gastrulation (Kimmel et al., 1995).

During the development of the blastula, the first differentiation between cell types occurs. At the surface, flattened epithelial cells form the enveloping layer (EVL) and cover the more loosely associated deep layer cells (DEL), which are multilayered compared to the monolayer of the EVL. No exchange of cells between the two layers is thought to occur and it is the movements of the DEL cells initiated at MBT which are required for epiboly, gastrulation and axis formation in the later embryo (Warga & Kimmel, 1990).

1.1.1.2 Epiboly and gastrulation

Elegant fluorescent cell labelling experiments have helped to uncover the mysteries of cell movements during embryogenesis, in assays which could not be performed in other vertebrate embryo models such as Xenopus or the mouse. At the end of the blastula stage, the blastoderm cells begin to intercalate and the blastoderm...
thins and becomes 'cup-shaped', covering the growing animal-ward bulge of the underlying yolk cell and indicating that epiboly is underway. By 5.2hpf, the blastoderm is halfway between the animal and vegetal poles, a stage known as 50% epiboly, and it is at this time that gastrulation begins. A thickened band appears more-or-less simultaneously around the circumference of the blastoderm, with DEL cells actively migrating towards the margin by means of blebbing and filopodial protrusions. Once at the margin, the earliest DEL cells to arrive reverse their direction and proceed back towards the animal pole in their new role as hypoblast cells in a layer beneath the epiblast. Those arriving later maintain a vegetal-wise progression (Warga & Kimmel, 1990). As epiboly proceeds and the blastoderm moves further towards the vegetal pole, so continues gastrulation, entailing that the hypoblast spreads from the margin to the animal pole.

Simultaneous to epiboly and gastrulation, a third morphogenetic movement occurs during the first 12 hours of embryonic development. Convergent extension causes DEL cells to move towards the presumptive dorsal side of the embryo and can be visualised relatively early by the presence of the shield, a thickening of the germ ring at around 50% epiboly which demarcates dorsal character (see fig. 1.3). In both the epiblast and the hypoblast layers, cells move from more lateral positions by medio-lateral intercalation and the subsequent narrowing and lengthening of the shield generates a well-defined embryonic axis within a couple of hours of shield formation (Warga & Kimmel, 1990). Concomitantly, the EVL cells move vegetally, via movements mediated by their contact with the YSL, and will differentiate into the periderm, a thin, flat cell layer swathing the outside of the whole embryo (Kimmel et al., 1995).

By 10hpf, the processes of epiboly and gastrulation are complete, with the bulk of the blastoderm having converged on the dorsal side of the embryo. Within a few minutes of yolk plug closure, the final stage of epiboly, the tailbud forms - a swelling at the most caudal end of the embryonic axis, the tailbud has been fate-mapped and found to contribute to the paraxial mesoderm and posterior spinal cord (Kanki & Ho, 1997).

1. Introduction

1.II.1.3 Axis specification and tissue restriction

The formation of axes and the correct distribution of tissue precursors are central to the processes of embryogenesis. As mentioned earlier, the animal-vegetal axis of the zebrafish embryo can be picked out almost immediately after fertilisation, by cytoplasmic streaming toward the animal pole; despite extensive mixing of cells
1. Introduction
during the blastula and early gastrula stages, there is a good correlation between
cells at the animal pole being found ultimately in anterior structures (Kimmel et al.,
1990). However, the exact time of specification of the dorso-ventral axis is less
well-defined and is thought to occur somewhat later. Early reports of a correlation
between early cleavage planes and dorso-ventral axis formation have been found to
be erroneous. However, the establishment of dorso-ventral asymmetry depends on
the transfer of components from the yolk into the embryo 'proper': an intact cortical
microtubule array leading from the yolk into the blastomeres is thought to mediate
this transfer, as disruption of such using nocodazole blocks the specification of the
dorso-ventral axis (Jesuthasan & Stahle, 1997). Likewise, there is a critical time
window, between 16 cells and MBT, for the sensitivity to lithium chloride treatment,
leading to the production of dorsalised embryos lacking any ventral specification and
again, this is thought to result from blocking a maternal signal (Stachel et al., 1993).

Kimmel et al. (1990) published the first detailed fate map of the embryo,
asserting that the first restriction of DEL cells occurred during the late blastula. In
the late gastrula, it was found that cells near the animal pole give rise to ectodermal
structures such as the brain and epidermis, whereas those nearer the margin produce
mesoderm and endoderm, such as the somitic musculature and the blood; dorsal cells
form dorsal and anterior structures like the notochord; ventral cells dorsal, ventral
and posterior structures like the pronephros (Driever, 1995; Kimmel et al., 1990).
More fine-grained analysis of the shield region, the presumptive zebrafish
homologue of the Xenopus organiser or Hensen's node of the chick, shows a group
of cells at the dorsal midline of the margin that develops exclusively as the
notochord and thus must play a key role in patterning the body axis and determining
its dorso-ventral polarity (Melby et al., 1996). A map of similar detail has been
produced for the developing neuroectoderm (Woo & Fraser, 1995), which shows a
latitudinal organisation for the nascent nervous system - those cells destined to
become forebrain, for instance, being positioned more towards the anterior pole than
those of the presumptive hindbrain.

1.1.1.4 Segmentation stages

From the formation of the tailbud at 10hpf, the succeeding 14 hours of
development are known as the segmentation stages. During this time, an array of
morphogenetic movements take place, bringing about the development of the
somites, the arisal of organ primordia and the lengthening of the body axis
accompanied by the first muscle contractions. The somites form at the rate of 2-3 per
hour and are initially cuboidal blocks of mesenchymal cells with an epithelial outer
layer, which take on their characteristic chevron-shaped appearance as the muscle pioneer cells elongate into the first muscle fibres spanning the length of each somite.

At the end of gastrulation, the epiblast has become exclusively ectodermal and one of its constituents, the neural plate, is well-defined and undergoes extensive shaping and specification into the central nervous system, such that by 24hpf, the eyes, neural tube and different regions of the brain are all clearly visible as distinct, differentiated structures. The regionalisation of the neural plate is underway even by the end of epiboly - expression of patterning markers such as *Krox20* and *pax2* delineate presumptive rhombomeres 3 and 5 and the midbrain-hindbrain boundary, respectively (Krauss et al., 1991; Oxtoby & Jowett, 1993). By 24hpf, the earliest steps in neurogenesis have taken place and a rudimentary axon scaffold is present in the anterior sections of the brain, as well as other primary sensory connections such as the trigeminal ganglia (Wilson et al., 1990).

1.II.1.5 Pharyngula and beyond

Over the course of the second day of development, the zebrafish embryo takes on the classic vertebrate embryo 'bauplan' and it is this time in which the embryo is seen to resemble that of other vertebrate species (Kimmel et al., 1995). The development of the notochord and somites is complete and the sculpting of the brain allows the five cerebellar lobes to be picked out. The branchial arches begin to differentiate and the individual elements can be identified from 72hpf onwards. The head lifts away from the yolk, which itself is becoming reduced in size as its store of nutrients is used up, and the pectoral and tail fins begin to develop, all in preparation for the free-swimming time ahead. The lateral line primordia migrate caudally along the trunk (fig. 1.3). Pigment cells develop and begin to distribute themselves in the characteristic longitudinal body stripes which give the fish its name; and the development of internal organs continues, so that a beating heart and circulating blood cells are clearly evident along with the not-as-easily seen developing gut, liver and kidneys (Kimmel et al., 1995).

In all, these changes entail that when the embryo hatches during its third day of development, it is becoming more and more equipped to cope with free-swimming and free-feeding that will become its lifestyle in the subsequent few days. Considering the above, it is perhaps easier to understand why the zebrafish has become such a popular choice for developmental biology. The transition from a ball of cells into a miniature adult fish over the course of a few days means that experiments can be carried out rapidly, and the optical clarity of the embryos allows
each stage of development to be monitored visually under a light microscope. Coupled with the array of in situ markers and antibodies available for marking different territories and cell types and the wide range of known mutants, the zebrafish certainly has come a long way from its home in the Ganges.

1.11.1.2 Mutagenesis screening

It was in 1981 that George Streisinger's landmark paper on the use of zebrafish was published in Nature. In it, he described a series of treatments by which large numbers of cloned, diploid embryos could be produced from homozygous mothers. Eggs were inactivated with genetically impotent, UV-treated sperm - initially, these haploids had been allowed to develop, but were found develop in a highly abnormal manner, thus not allowing any genuine mutant features to be picked out. The paper described two methods of circumventing this, by either applying hydrostatic pressure or heat-shocking the activated eggs, either of which blocked the partitioning of the duplicated maternal haploid chromosome complement into daughter cells and thus produced embryos which were homozygous diploids (Streisinger et al., 1981). This method allowed the identification of recessive lethal mutations and the rough mapping of genetic distance from the centromere, based on recombination frequencies, but was found to be dissatisfactory on account of the high background level of abnormal development caused by damage to the eggs, and the mis-identification of mutations in genes distant from the centromeres caused by crossing-over (Driever et al., 1994).

An alternative approach to the identification of recessive mutations was developed, based on the large-scale screens carried out in Drosophila (Nusslein-Volhard & Wieschaus, 1980). Using the mutagen ethyl-nitrosourea (ENU), it was found that spermatogonia of founder male fish could be mutagenised in a way to produce an average of one 'hit' per haploid genome - this technique induces point mutations and so is preferable to X- or γ-ray mutagenesis in which whole sections of chromosome can be broken or lost (Grunwald & Streisinger, 1992a; Grunwald & Streisinger, 1992b); additionally, the whole length of each chromosome is supposedly equally susceptible to mutagenesis, eliminating any effects of centromeric distance in the recovered progeny (Driever et al., 1994). The mutations are bred to homozygosity in a three-generation scheme (fig. 1.4) and the presence of recessive mutations can be seen in the F3 progeny, where 25% of a clutch will show the phenotype. This was found to be a reliable and efficient method of generating unambiguous mutants (Solnica-Krezel et al., 1994) and several, large-scale screens
Fig. 1.4 Mutagenesis screening produces homozygous mutant zebrafish embryos

Male fish are treated with the mutagen ENU and crossed to wild-type females. In the F_1 generation, each individual is heterozygous for a different mutated gene. Crossing of these fish with wild-type females results in an F_2 generation where 50% of the fish in a given family carry the same mutation. The F_2 fish are incrossed and their progeny examined for developmental abnormalities - embryos homozygous for an induced mutation will be found in 25% of these sib matings. Wild-type alleles are denoted by a plus sign; induced mutations by an additional asterisk.

(Adapted from Wolpert et al, 1998)
Mutagen treatment of spermatogonia

Gametes +

Possible sibling matings

F₁

F₂

F₃
have been carried out in this manner, facilitated by the short generation time and the ease of culture of the fish (Driever et al., 1996; Haffter et al., 1996). Such screens would be unthinkable for practical and genetic reasons in the mouse or *Xenopus*, whereas the *Drosophila* screens may be informative for the identification of conserved genes acting on essential processes in development but provide little scope for the description of genes involved, say, in the specification of neural crest, a vertebrate 'invention' which they do not possess.

The fruits of one of the largest zebrafish screens were described in a special edition of the journal Development in 1996 and it was clear to see how mutations could be found in processes affecting all levels of development, from gastrulation defects (Hammerschmidt et al., 1996) to the development of the viscera (Pack et al., 1996). The majority of these mutants were identified by their morphology alone, thus claiming another hit for the optical clarity of zebrafish development. It was with this in mind that an ENU screen was carried out at in the zebrafish group at University College London and there is the description and characterisation of one such recovered mutation in Chapter 5.

1.II.1.3 Future directions for the zebrafish

The world of zebrafish research is progressing from strength to strength. A number of genetic maps are available and the refinement of these is allowing the accurate mapping of both genes and mutants - both forward and reverse genetics are relatively simple in the zebrafish (Gates et al., 1999; Knapik et al., 1996). There is also a rise in the existence of resources such as the expressed sequence tag (EST) database, in which genes of interest can be searched for, often on the basis of homology to those in other species and obtained as DNA clones for further and more extensive characterisation and mapping (Kelly et al., 2000). The 'classic' techniques of DNA and RNA injection into early embryos (Holder & Xu, 1999) have also been joined recently by the morpholino (MO) injection (Nasevicius & Ekker, 2000), in which oligonucleotides corresponding to the 5' extent of a transcript can be injected to block its function, putatively by the formation of a stable helix which blocks the translational start site of the mRNA. Although still in its infancy, this technique has already been shown to specifically phenocopy known mutants such as *notail* and *sparse* (Ekker, 2000), generating so-called 'morphants'. This technique may become of use clinically, in the development of zebrafish models of human disease.

There is also a mounting interest in transgenesis. Fish have been created which express green fluorescent protein under the control of specific neural
promoters (L. Roth, unpublished) and there is the construction of the UAS-Gal4 system based on that in *Drosophila* (Brand & Perrimon, 1993), both of which will help to broaden our knowledge of vertebrate development and open up new avenues of research.

1.II.2 Summary

The zebrafish has come a long way in the past 20 years from being a novel vertebrate model favoured by few to a widely-used developmental paradigm with proven worth. The ability to be able to carry out both genetics and physical techniques such as grafting and single-cell labelling, accompanied by the ease with which the embryos can be cultured and manipulated, sets the zebrafish apart from the mouse and *Xenopus* in terms of versatility.
1.2 The project

The work described in this thesis is divided into two sections. The first deals with the role of the Rho GTPases during vertebrate development, using the zebrafish embryo as a model system. The cloning and expression of two zebrafish Rho family homologues is described, \textit{zRhoA} and \textit{zRacl}, with detailed expression patterns being presented for both during the course of embryogenesis and in the adult brain. Following on from this, chapter 4 addresses the possible function of RhoA during early development. RNA encoding the wild-type, constitutively active and dominant negative forms of human \textit{RhoA} is injected and the resultant phenotypes are analysed, with comparison being made between the phenotype seen when constitutively active \textit{RhoA} is injected and the phenotype of the \textit{silberblick} mutation. There is also an investigation into the abrogation of Rho function in the embryo brought about by the introduction of RNA encoding C3 exoenzyme, a pan-Rho blocking agent.

The second section of this thesis describes the \textit{gimpy} mutant, a strain which was isolated from an ENU mutagenesis screen carried out at University College London. The predominant phenotypes seen in these embryos are their dwarf-like appearance with their shortened embryonic axis, and their lack of a differentiated notochord. The underlying bases for these are investigated, including the \textit{in vivo} examination of morphology, \textit{in situ} and immunohistological analysis and examination of the embryonic ultrastructure. There is also a discussion of the complementation of \textit{gimpy} with other, known dwarf-like mutants and candidate genes are speculated upon.
CHAPTER 2

Materials and Methods

2.1 Maintenance of Zebrafish

A breeding colony of zebrafish were maintained at 28.5°C on a 14 hour light/10 hour dark cycle (Westerfield, 1993). Embryos were collected from natural spawnings using a tea strainer and staged according to (Kimmel et al., 1995). Embryos were generated from the wild-type lines utwil, *AB, Tübingen and Tup longfin. The single recessive lethal mutants, *gimpy* and *silberblick* (Heisenberg et al., 1996) were also used.

Embryos were cultured in fish system water supplemented with Methylene Blue, with non-pigmented embryos being generated by the addition of 0.2 mM phenylthioarbamide to this medium.

2.2 Observation of Live Embryos

Embryos were observed in fish system water under a Leica dissecting microscope. Dechorionation was carried out manually using #5 gauge watchmaker's forceps. When required, embryos were anaesthetised with 0.02% tricaine (3-aminobenzoic acid ethylester) and immobilised for viewing in 3% methyl cellulose in embryo medium.

2.3 Molecular Biology Techniques

Standard molecular biology techniques were carried out according to Sambrook et al. (1989). For large scale DNA preparations, plasmids were transformed into *E. coli* (either XL-1 Gold or JM109 strains) and cultured in 100 ml LB medium supplemented with ampicillin (Sigma; 80 µg/ml). DNA was purified using a Qiagen Midi prep column (Qiagen).

Restriction enzyme digests were carried out in 20-100 µl using an appropriate 1x enzyme buffer and 2-5 units of enzyme (Promega) per 1 µg DNA. Digests were carried out at 37°C and checked on 0.8-1.2% agarose TBE or TAE gel by electrophoresis. Purification of DNA was carried out by 1x phenol extraction, 2x phenol:chloroform extraction and 1x chloroform extraction followed by precipitation. DNA was precipitated with 0.1 volume 3 M NaAc and 2.5 volumes.
100% ethanol at -80°C for 30 minutes. Precipitated DNA was microfuged for 15 minutes, the pellet was washed in 70% ethanol, air dried and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), containing 40mg/ml RNAseA where appropriate.

2.3.1 cDNA cloning of zebrafish small GTPases

2.3.1.1 Cloning of zebrafish RhoA

A radiolabelled DNA probe was generated from a human Rho cDNA clone (gift; Kate Nobes, LMCB) using the Prime-A-Gene kit (Promega) and cleaned using a Sephadex (Sigma) G-50 column. The probe was used to screen a zebrafish somitogenesis stage cDNA library (gift; David Grunwald) titred to achieve 1x10^6 clones over 25 150mm filters. Filters were pre-hybridised in 50% formamide, 5x Denhardts, 6.5x SSC, 2% SDS, at 42°C for at least 2 hour. Hybridisation was at 42°C overnight, with the addition of heat-denatured probe and 100ng/ml sonicated herring sperm DNA. Low stringency post-hybridisation washes were performed in 2x SSC, 0.1% SDS at 42°C for 6x 15 minutes, then 2x 30 minutes. This procedure was repeated twice to progressively purify positive clones and from a tertiary screen, 24 positive Bluescript phagemids were rescued using the λZAP II system - Southern blotting revealed seven of these to be false positives however. Of the four positive clones ultimately sequenced, one was chosen as a representative zebrafish Rho clone, possessing the full-length coding sequence flanked by approximately 40bp of 5' UTR and 750bp of 3' UTR, giving a total insert size of 1.35kbp.

2.3.1.2 Cloning of zebrafish Rac1

PCR primers to conserved regions within the Rac protein (gift; Kate Nobes - see Appendix 3 for sequences) were used in a low-stringency polymerase chain reaction (PCR) amplifying a zebrafish embryonic cDNA library (70% epiboly - 18 somites; constructed by Anukampa Barth, Zebrafish Group, U.C.L.). 100-120 pmols of each degenerate primer, and 70ng of the diluted cDNA library were used in PCR reaction mixtures of 50 µl volume. 40 amplification cycles (94°C for 45 seconds; 48°C for 2 minutes; 72°C for 45 seconds) were carried out. The resultant 200-300bp products were cloned into the pCRII-TOPO vector using the TA-cloning kit (Invitrogen). Twenty clones were sequenced, of which seven were found to be homologous, to varying extents, to vertebrate Rac1. The clone with the greatest homology was used in the synthesis of a radiolabelled probe and a low-stringency
hybridisation to a zebrafish somitogenesis stage cDNA library was carried out as detailed in section 2.3.1.1.

From a tertiary screen, twenty-seven positive clones were isolated, of which four of the fourteen clones found to be 'true' positives on Southern blotting were sequenced. Although not full-length, the clone chosen for further work possessed the greatest degree of homology of these four to the vertebrate Rac1 sequence, consisting of a 546bp coding sequence and around 1kbp of 3' UTR.

2.3.1.3 Nomenclature

The DNA sequences of the clones amplified from the successive rounds of library screening were processed using the Gene Jockey and MacVector sequence analysis software to determine their open reading frames and the amounts of UTR present. The sequences were compared to those in the NCBI database using the BLASTx algorithm and the resulting hits used in the nomenclature of the clones - thus, \( z\text{RhoA} \) and \( z\text{Racl} \) were assigned to the clones isolated from the first and second rounds of library screening respectively.

2.3.2 Southern Blotting

The clones to be tested were digested with restriction enzymes appropriate for the excision of the insert and were separated electrophoretically on a 0.7%-1%% TAE agarose gel. The gel was trimmed down, notched for later orientation, measured and photographed. It was then treated in denaturing solution, 0.5M Tris pH 7.2, 1M NaCl for 15 minutes and then a further 20 minutes after a change of solution. Meanwhile, a nylon membrane (Hybond-N; Amersham) was cut to the same size, wetted in distilled water and soaked in transfer solution, 0.4N NaOH, 1M NaCl for five minutes. The gel was inverted on a support covered in 3M Whatman paper, the filter placed on top and a weighted stack of 3M paper and paper towelling built up. The apparatus was dosed with transfer solution and left for 12-24hr. The apparatus was disassembled and the filter soaked in denaturing solution for 15 minutes. After drying and UV-light crosslinking, the filter was hybridised at the same stringency as the original library screening had been carried out (see section 2.3.1.1) and exposed to X-ray film (Kodak X-OMAT XAR-5) at 80°C for 1-5 days. The film was developed and the banding pattern compared to the original gel photograph to determine the position of the positive clones.
2. Materials and Methods

2.4 In Situ Hybridisation

2.4.1 Synthesis of antisense RNA probes for in situ hybridisation

Templates for synthesis of antisense RNA probes were generated by linearising the clone at the 5' end with an appropriate restriction enzyme. Following phenol:chloroform extraction and precipitation (section 2.3.) the DNA was resuspended in low TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). 1 µg of the linear DNA was used for the in vitro transcription reaction, in which the RNA probe was labelled with digoxygenin-11-UTP. A 20 µl reaction was set up in 1x transcription buffer (200 mM Tris-HCl, pH 7.5, 30 mM MgCl₂, 10 mM spermidine, 50 mM NaCl), 10 mM dithiothreitol (DTT), NTP-digoxygenin mix (1 mM ATP, CTP, GTP, 0.65 mM UTP, 0.35 mM UTP-digoxygenin), 40 units RNasin, 10 units T3/T7/SP6 RNA polymerase. The transcription mix was incubated at 37°C for 2 hours before 1 unit of DNase was added to remove the template. The DNase reaction was stopped after 15 minutes at 37°C by the addition of 1 µl 0.5 M EDTA, pH 8.0. The RNA was precipitated with 0.5 volumes 1M NH₄Ac, 2.5 volumes 100% ethanol at -20°C for 30 minutes, washed in 70% ethanol and air dried. The RNA pellet was resuspended in 20 µl water and made up to 100 µl with prehybridisation mix (50% formamide, 5x SSC, 50 µg/ml heparin, 500 µg/ml torula RNA, 9.2 mM citric acid, 0.1% Tween-20), and standardly used at a dilution of 1 in 200.

2.4.2 Whole-mount in situ hybridisation

The whole-mount in situ hybridisation protocol was based on that of Thisse et al., (1993). Embryos were fixed in 4% paraformaldehyde in PBS, pH 7.4 (4% PFA), overnight at 4°C or for 3-4 hours at room temperature. Embryos younger than 24 hours post fertilisation (hpf) were dechorionated following fixation, and older embryos were dechorionated prior to fixation. Embryos were rinsed in PBT (PBS, 0.1% Tween-20), dehydrated through a methanol series (75% PBT:25% methanol; 50% PBT:50% methanol; 25% PBT:75% methanol) and held in 100% methanol at -20°C for at least 30 minutes.

Embryos were rehydrated with 5 minute washes of 75% methanol:25% PBS, 50% methanol:50% PBS, 25% methanol:75% PBS, followed by 4 washes in PBT, before prehybridisation at 65°C for 2 hours in prehybridisation mix. Embryos older than 100% epiboly (10 hpf) were treated with 10 µg/ml proteinase K at 20°C for 3-30 minutes depending on age, rinsed twice in PBT and refixed in 4% PFA for 20 minutes at room temperature. After refixation embryos were washed 5x 5 minutes in
PBT before prehybridisation. The RNA probes were added and hybridised overnight at 65°C.

Post hybridisation washes were carried out at 65°C. Embryos were rinsed in prehybridisation mix, and then 15 minute washes with 75% prehybridisation mix:25% 2x SSC, 50% prehybridisation mix:50% 2x SSC, 25% prehybridisation mix:75% 2x SSC, 100% 2x SSC. This was followed by 2x 30 minute washes in 0.2x SSC.

Embryos were washed into antibody block at room temperature. 5 minute washes were carried out in 75% 0.2x SSC:25% MAB (0.1 M Maleic Acid, 0.15 M NaCl, pH 7.4), 50% 0.2x SSC:50% MAB, 25% 0.2x SSC:75% MAB, and 100% MAB. Embryos were blocked in 2% Boehringer block (Boehringer Mannheim) in MAB for 2 hours. The embryos were incubated in anti-digoxigenin-alkaline phosphatase Fab fragments (1 in 5000) overnight at 4°C or 3-4hr at RT.

The antibody was washed off 8x 15 minutes in MAB at room temperature. The embryos were equilibrated 3x 5 minutes in developing buffer (0.1 M Tris, pH 9.5, 50 mM MgCl₂, 0.1 M NaCl, 0.1% Tween-20). The embryos were developed in BM Purple Substrate (Boehringer Mannheim) in the dark. The colour reaction was stopped by rinsing 3x PBT, and refixing for 2h at room temperature. Embryos were cleared through a glycerol (Sigma) series (30%; 50%; 70% glycerol in PBS) and stored and photographed in 70% glycerol. Optical sections of larval-stage embryos were taken by hand using tungsten needles and mounted again in 70% glycerol under coverslips.

2.4.3 In situ hybridisation of adult brain tissue

Wild-type adult zebrafish were euthanased and their heads removed into 4% PFA. After overnight fixation at 4°C, the brains were dissected out and fixed for a further few days in 4% PFA at 4°C. After rinsing in PBS, the brains were orientated and embedded in 5% low-melting point agarose dissolved in PBS. Sections 300-500µm in thickness were cut using a vibratome (Campden Instruments Ltd.) and then taken through the in situ protocol as above, the only modifications being the reduction of the Proteinase K incubation to 3 minutes and an extra Hyb rinse prior to the overnight probe incubation. After the colouration reaction in BM Purple substrate, the brain sections were washed several times in PBT and dehydrated through a methanol: PBT series (5-10 minutes each in 25:75; 50:50; 75:25 methanol: PBT; 30 minutes in 100% methanol.) Following rehydration, the sections were
cleared through a glycerol series and mounted under coverslips in 70% glycerol for photography. (This procedure was also used, without the dehydration steps, for trunk sections of 24-72hpf embryos, for which 100-200μm sections were cut.)

2.4.4 cDNAs used for in situ hybridisation

The following zebrafish cDNA clones were used as templates to synthesise RNA probes for expression analysis: krox-20 (Oxtoby & Jowett, 1993), pax-2 (Krauss et al., 1991a), shh (Krauss et al., 1993), ntl (Schulte-Merker et al., 1994a; Schulte-Merker et al., 1994b), gsc (Stachel et al., 1993), her-1 (Muller et al., 1996), MyoD (Weinberg et al., 1996), emxl (Morita et al., 1995), hggI (Barth, A. K., unpublished), papc (Yamamoto et al., 1998), pax6 (Krauss et al., 1991b), lim3 (Glasgow et al., 1997) and the previously mentioned zRhoA and zRacI.

2.4.5 Cryosectioning of stained embryos

Cleared embryos were taken back through a glycerol series and rinsed several times in PBS. Embryos were then embedded in TissueTek sectioning medium (Sigma) and left to harden at -80°C for 30 minutes. Embryos were mounted on chucks using TissueTek and 14-20μm sections cut on a Cryocut 1800 (Leica) cryostat at -20°C. Sections were collected on TESPA-coated slides (1% TESPA in acetone for 30 minutes, followed by 2 acetone washes; Sigma), washed briefly in PBS to remove sectioning medium and then cover-slipped over 70% glycerol for observation and photography.

2.5 Northern Blotting

2.5.1 Total RNA extraction

Around 50 embryos for each stage required (50% epiboly, 6-10 somites, 24hpf, 48hpf, 72hpf) were collected, dechorionated and, where necessary, anaesthetised and dissected. The embryos were kept on ice to limit RNA degradation and macerated using the Qiashredder (Qiagen) apparatus. Total RNA was extracted from this mixture using the RNeasy kit (Qiagen), eluted in 50-60μl RNase-free water and stored at -80°C. The amount of total RNA present in each sample was determined using a spectrophotometer and it was assumed that 1% of this amount would be mRNA species.
2.5.2 Northern Blotting

2.5.2.1 Preparation of the gel and samples

A gel was prepared using 1% agarose, gel running buffer (5X stock: 0.1M MOPS pH7, 40mM NaAc, 5mM EDTA pH8) and filter-sterilised formaldehyde to give a final concentration of 2.2M. Once polymerised, the gel was pre-run in 1X running buffer for 5 minutes at 5V/cm. The samples were prepared by taking a known amount of total RNA (1μg, as determined by the spectrophotometric measurements) and incubating it in a total volume of 20μl (1X gel running buffer, 17.5% formaldehyde and 50% filter-sterilised formamide) at 65°C for 15 minutes and then snap-cooling on ice. Before loading onto the gel, 2μl sterile loading buffer (50% glycerol, 1mM EDTA pH8, 0.25% each bromophenol blue and xylene cyanol FF) was added and the samples centrifuged briefly. The gel was run at 3-4V/cm for several hours with periodic recirculation of the running buffer until the bromophenol blue dye front had migrated approx. 8cm.

2.5.2.2 Transfer of RNA onto and hybridisation of the filters

The gel was rinsed several times in RNAse-free water to remove the formaldehyde present and then soaked in 20X SSC for 45 minutes. After measurement and trimming, a stack was built up consisting of the gel on a 3M covered support bathed in 20X SSC. Nylon filters, prewetted in 20X SSC, were placed onto the gel and a stack of 3M and paper towels assembled on top of this and weighted down. Transfer was allowed to continue for 6-18 hours before the assembly was dismantled and, after marking the position of the gel slots onto them, the filters were washed in 6X SSC for 5 minutes at room temperature, dried and the RNA cross-linked onto them using UV light.

To ensure the presence of RNA on the filters, they were soaked in 5% acetic acid for 15 minutes at room temperature and then incubated for 5-10 minutes in 0.5M NaAc pH5.2, 0.04% methylene blue. After several washes in sterile water, an array of stained bands was clearly visible in each lane and the filters were photographed.

Prehybridisation was carried out at 65°C for 2-4hr in hybridisation mix (50% formamide, 5X SSC, 1% blocking powder (Boehringer), 0.5% SDS) and denatured, digoxygenin-labelled probe (prepared as for in situ hybridisation) was included in the overnight hybridisation at a concentration of 20-30 ng/ml. The following day, the
2. Materials and Methods

Filters were washed 2x 5 minutes at room temperature in 2X SSC, 0.1% SDS; then 2x 30 minutes at 65°C in wash buffer (0.1X SSC, 0.5% SDS). After rinsing briefly in wash buffer, the filters were incubated in maleic acid blocking solution for 2 hours at room temperature, followed by 30-60 minutes in anti-digoxygenin-alkaline phosphatase F\textsubscript{ab} fragments, 1:10000 dilution in maleic acid blocking solution. Once the antibody incubation was complete, the filters were washed 2x 20 minutes at room temperature in wash buffer, followed by 5 minutes in the BCL colouration buffer. The BM purple substrate was then used for the visualisation of the bound antibody, as per the \textit{in situ} hybridisation reaction. The filter were photographed and analysed for the different \textit{zRho} and \textit{zRac} species present.

2.6 Injection of mRNA into Early Stage Embryos

2.6.1 Cloning of \textit{hRho} and \textit{C3} constructs into expression vectors

Initially, the \textit{hRho} and \textit{C3} inserts were cloned into the pRK5-MYC vector which lacked any transcriptional start sites for \textit{in vitro} RNA synthesis. Using primers specific for the 5' and 3' terminal sequences for Rho and C3 respectively (see Appendix 4), a PCR based approach was used to subclone the inserts into the pCS2-MT vector, which also enabled the inserts to be labelled with 6 myc epitopes at the 5' end. The hRho inserts (wild-type - G14; dominant negative - N19; constitutively active - V14) were amplified in a volume of 25\textmu l using the KOD-dash polymerase (Toyobo) with 30 cycles (30 seconds 94°C; 45 seconds 60°C; 1 minute 72°C), with a similar protocol being used for the C3 insert - for this however, the annealing temperature was reduced to 42°C. After incubation for 10 minutes at 72°C with Taq polymerase to generate the requisite overhanging ends, the inserts were subcloned into the pCR-TOPO vector using the TA cloning kit (Invitrogen). These constructs were amplified and the inserts excised using the EcoRI and XbaI sites added in the PCR reaction. Finally, these inserts were cloned into the pCS2-MT vector which had been pre-digested with these same enzymes to generate compatible, 'sticky' ends. The orientation and accuracy of the inserts was verified by sequencing (ABC, Imperial College.)

2.6.2 Other templates used for RNA synthesis

The pCS2-eGFP (Turner and Rupp, 1995) construct was used as a control template for the \textit{in vitro} transcription reaction and as a control RNA species for injection into embryos.
2.6.3 **In vitro transcription of mRNA for injection**

*In vitro* transcriptions were carried out using the Ambion Megascript Kit (Ambion). In a total reaction volume of 20 µl, 1 µg linear DNA template, 1x transcription buffer, 5 mM ATP, 5 mM CTP, 5 mM UTP, 1.5 mM GTP, 6 mM 7mG(5') ppp(5')G sodium salt (capGTP), and 2 µl SP6 RNA polymerase enzyme mix were used. Transcription reactions were incubated for 3 hours at 37°C before the DNA template was removed by the addition of 1µl DNaseI to the reaction and a further incubation of 15 minutes.

The reaction was terminated by the addition of 115 µl dH₂O and 15 µl 3 M NaAc. The RNA was extracted twice with phenol:chloroform and then precipitated by the addition of one volume of 100% isopropanol and incubating at -80°C for 15-30 minutes. The RNA was microfuged for 15 minutes at 4°C, washed in 70% ethanol, and resuspended in 200 µl dH₂O before being cleaned and concentrated using a microconcentrator (microcon 100, Amicon) and microfusing at 4°C. The final concentration was determined spectrophotometrically and the RNA was diluted, aliquotted and stored at -80°C.

2.6.4 **Injection of mRNA into early stage embryos**

1-8 cell stage embryos were aligned along the edge of a glass slide with minimal fish water covering them. RNA was injected through the chorion into one cell of the embryo, in a volume of approximately 200 pl using a glass capillary needle attached to a Picospritzer. The injected embryos were left to develop in fish tank water at 31°C, and unfertilised embryos were discarded during blastula stages. The number of remaining embryos was noted, and a number of uninjected embryos maintained separately as controls.

The amount of RNA injected was 25-200 ng/µl for the Rho constructs, 10-100ng/µl for the C3 construct and 95 ng/µl for pCS2-eGFP. The distribution of injected RNA *in vivo* was determined by post-fixation immunostaining for the Myc epitope (see below.)

2.7 **Analysis of GFP Expression**

Control embryos injected with the eGFP RNA were assayed for its expression by examination of live embryos under a fluorescence dissecting microscope (Leica).
2.8 Immunohistology on Whole-Mount Embryos

2.8.1 Fixation of tissue

Whole embryos and larvae younger than 48h were fixed (in their chorions if younger than 22 somites, otherwise dechorionated) in 4% paraformaldehyde (4% paraformaldehyde in PBS) for 3-4 hours at room temperature or overnight at 4°C. Larvae older than 48h were first anaesthetised in 0.03% MS222 (3-amino benzoic acid ethyl ester, Sigma) before fixation in 2% TCA (tri-chloroacetic acid; Sigma) for 3 hours exactly at room temperature. After fixation, embryos were washed 3 times in PBS and then processed as below.

2.8.2 Labelling

Embryos were rinsed several times in PBTx (0.8% Triton-X-100 in PBS) at room temperature, unless the zn-5 primary antibody was to be used, in which case 1% dimethylsulfoxide (DMSO) was added to the PBTx and the embryos permeabilised for 7 minutes in acetone at -20°C. Those older than 48h were permeabilised by incubation in 2.5 mg/ml trypsin solution for 4 minutes on ice followed by extra PBTx washes. Embryos were then incubated in 10% foetal calf serum in PBTx for 1 hour at room temperature in order to block non-specific binding of the antibody, before being left overnight at 4°C in a solution of the primary antibody diluted in 1% foetal calf serum in PBS. The following day, the embryos were washed over several hours with several changes of PBTx and incubated in the secondary antibody (Sigma) diluted in 1% foetal calf serum in PBTx overnight at 4°C - in general, secondary antibodies were used at a 1:200 dilution. The embryos were again washed several times in PBTx over a number of hours before processing and visualisation of the bound antibodies. For horseradish peroxidase (HRP)-conjugated antibodies, the embryos were incubated in 2mM diaminobenzidine (DAB; Sigma) in PBS for 10 minutes at room temperature prior to the addition of 1-2µl 3% H₂O₂ (Sigma). Once the reaction had been deemed to have gone to completion, as seen by the appearance of the brown reaction product, the reaction was stopped by PBS washes and the embryos post-fixed in 4% paraformaldehyde for 2 hours at room temperature. Embryos were cleared through a glycerol series and stored in 70% glycerol for observation, dissection and microscopy.
2. Materials and Methods

Primary antibodies used were anti-acetylated tubulin (Wilson et al., 1990) (Sigma), 1:1000; anti-Myc (Sigma), 1:750; anti-PH3 (Delaney, D. PhD thesis 1999) 1:1000; HNK-1 (Wilson et al., 1990), 1:2000; pan-myosin, (Simon Hughes, gift), 1:10; zn-5 (Trevarrow et al., 1990), 1:50; 3A10 (Furley et al., 1990), 1:250. Secondary antibodies were anti-mouse IgG for all reactions except HNK-1, for which anti-mouse IgM was used.

2.9 Haematoxylin Staining

The tissue to be stained was rehydrated through a reverse glycerol series where necessary and then rinsed in PBS several times. The tissue was incubated for 10 minutes at room temperature in Mayer’s haematoxylin solution (Sigma) and washed in copious, running tap water for a minute prior to being differentiated in 1% hydrochloric acid, 70% ethanol for 10 seconds. After a further 5 minutes rinsing under tap water, the tissue was cleared through glycerol and mounted under coverslips for photography.

2.10 Acridine Orange Staining

Live embryos of 24-28hpf were incubated in 500ng/μl Acridine Orange (Sigma) solution for 30 minutes at room temperature. After a few rinses in embryo medium, the embryos were examined under a fluorescence compound microscope (Nikon), with any dead/dying cells being fluorescently labelled.

2.11 Alcian Blue Staining

Alcian Blue staining was performed adapting the protocol developed by Schilling et al., (1996). 5dpf embryos were fixed for 4 hours at room temperature in 4% paraformaldehyde. After washing in PBS, they were stained for 24 hours in 0.1mg/ml Alcian Blue (Sigma) in a solution of 80% ethanol, 20% glacial acetic acid. The embryos were then rehydrated slowly (1-3 hours for each step) through an ethanol series - 70%, 50%, 30% and distilled water. The overlying muscle was removed using 5mg/ml trypsin in a solution of 30% saturated sodium tetraborate until the cartilage became clearly visible. The embryos were then differentiated at 37°C in a 1% hydrogen peroxide, 1% ammonia solution with frequent monitoring. Finally, the tissue was cleared in a 0.5% potassium hydroxide/glycerol series and the embryos mounted and photographed in 70% glycerol.
2.12 Electron Microscopy

Embryos were fixed for 48hr at 4°C in 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer. After several rinses in 0.1M sodium cacodylate buffer, the embryos were fixed overnight at 4°C in 2% osmium tetroxide in cacodylate buffer and then washed in buffer alone before being dehydrated through an ethanol series (10-15 minutes each in 25%, 50%, 75%, 90% ethanol, followed by 2x15 minutes in 100% ethanol.) The ethanol was exchanged for propylene oxide (2x15 minutes; BDH) and gradually taken into resin (6g agar100 resin; 4g dodecenylsuccinic anhydride hardener; 2.5g methyl nadic anhydride hardener), being incubated on a roller in 1:1 resin: propylene oxide for 30 minutes and 3:1 for 1 hour, followed by pure resin for up to 24 hours. Fresh resin was then made, with the addition of 0.38g benzylidimethylamine accelerator and the embryos embedded in this in coffin moulds for 48 hours at 60°C. Once hardened, the blocks were trimmed and ultrathin (50-100nm) sections cut using a diamond knife on an ultratome (LKB Bromma). Sections were mounted on 3mm copper grids (Athene new 200) and stained with 2% uranyl acetate for 30 minutes before rinsing thoroughly with sterile water. After mounting on Snaptek gridsticks, the sections were stained for 5 minutes with Reynold’s lead citrate, rinsed, dried and visualised under transmission electron microscopy.

2.13 ENU Mutagenesis Screen

2.13.1 ENU mutagenesis

Mutagenised spermatogonia were generated by means of ethyl-N-nitrosourea (ENU; Sigma) treatment of adult male fish of the tupl\textsuperscript{f} genetic strain. Fish were mutagenised by placing them into a 3mM, aqueous solution of ENU for three 1 hour intervals over the course of one week. Three weeks after this treatment, these males were crossed to wild-type females at weekly intervals and their progeny raised i.e. those offspring originating from mutagenised premeiotic germ cells. Each mutagenised male's offspring were maintained as a separate line so that any subsequent mutations arising could be traced back to a particular founder male. The families thus arising were then incrossed to generate F\textsubscript{2} lines whose offspring (the F\textsubscript{3} generation) would be screened for the presence of recessive mutations, with 12 mating pairs being set up for each F\textsubscript{2} family to ensure statistically the recovery of any recessive mutation present.
2. Materials and Methods

2.13.2 Mutant analysis

$F_2$ fish were maintained on a 9.30am light cycle and females tended to spawn at this time. Clutches of eggs were collected, cleaned and screened cursorily for epiboly defects. At 24hpf, the embryos were rigorously screened under a dissecting light microscope (Leica) for the presence of morphological defects obeying Mendelian 3:1 ratios with respect to the number of embryos in the clutch. At 48hpf, embryos were fixed in TCA and treated with the anti-tubulin antibody to look for irregularities in the central nervous system. Any pair of fish with mutant offspring were maintained separately from their sibs and screened again the following week to check for consistency of the mutation. The remainder of the family were also rescreened to find additional carriers and carriers were outcrossed to wild-type fish to establish the mutation as a novel genetic line.
CHAPTER THREE

The Cloning and Expression of Two Members of the Zebrafish Rho GTPase Family

3.1 Introduction

Much of the work thus far carried out on the Rho family has been performed \textit{in vitro} using cell culture. Members of the family have been cloned and analysed in \textit{C. elegans} (Chen \& Lim, 1994), \textit{Drosophila} (Hariharan et al., 1995), chick (Malosio et al., 1997) and \textit{Xenopus} (Wunnenberg-Stapleton et al., 1999) but as yet, none have been described in the zebrafish. The work contained in this chapter describes the cloning and expression analysis of two, hitherto unknown, Rho GTPases which I have designated as \textit{zRhoA} and \textit{zRacl}.

3.2 Results

3.2.1 Cloning of zebrafish \textit{zRhoA}

3.2.1.1 Isolation of a \textit{zRhoA} clone

In order to isolate zebrafish Rho family clones, a low-stringency screen was carried out of a zebrafish late-somitogenesis stage \textit{\lambda}ZAP library, using a radiolabelled full-length human \textit{RhoA} probe of approximately 570bp (gift, Kate Nobes, LMCB.) After three rounds of progressive purification, twenty-four positive clones were isolated - however, Southern blotting revealed some of these to be false positives. Of the seventeen remaining clones, four were submitted for sequencing and BLAST database comparison showed all of these to be \textit{RhoA} homologues. Clone 23A11 was chosen for all further analysis, as it possessed the greatest degree of homology to RhoA clones from other species and was named \textit{zRhoA} on account of this homology.

The isolated \textit{zRhoA} clone was found to possess a 1.35kbp insert, consisting of the entire \textit{zRhoA} open reading frame (579bp - see Appendix 2) flanked by about 40bp of 5' UTR and 750bp 3' UTR. A consensus Kozak sequence was also present: A at -3 and G at +4 relative to the predicted ATG start codon (Kozak, 1987). The encoded DNA sequence was processed using the Gene Jockey sequence analysis program to deduce the corresponding amino acid sequence (Fig. 3.1) and the protein
Fig. 3.1 Nucleotide sequence and deduced amino acid sequence of the zebrafish $zRhoA$ clone

The insert contains the entire open reading frame for the zebrafish $RhoA$ gene, including the 3' stop codon at position 580. The sequence is numbered relative to the start of the deduced coding sequence.
was seen to possess the characteristic Rho family C-A-A-X terminal motif required for post-translational modification (Adamson et al., 1992).

3.2.1.2 **Evolutionary comparisons with RhoA from other species**

Comparison of the inferred amino acid sequence to those known from other species using the ClustalW software program showed a very high degree of conservation across evolution (fig. 3.2) from higher vertebrates, (97% identity between the rat and zebrafish sequences) to invertebrates (88% identity between the *Aplysia* and zebrafish sequences.) The five regions critical for Rho function (Ridley, 1997) (indicated in fig. 3.2) are all highly conserved between the proteins. Accordingly, there was also considerable sequence identity at the DNA level - between 70 and 78%, depending on the species compared.

3.2.2 **Expression of zRhoA during development**

3.2.2.1 **Expression during early embryogenesis**

The zRhoA transcript is expressed ubiquitously at all stages tested by in situ hybridisation up to and including the tailbud stage in the first twelve hours of embryogenesis (fig 3.3a-d). The expression seen prior to mid-blastula transition (Kane & Kimmel, 1993), the period at which zygotic transcription is initiated, suggests that there is a maternal zRhoA contribution to the early embryo. Levels of expression remain high in all cell layers throughout epiboly and gastrulation (fig. 3.3c, d).

3.2.2.2 **Expression during segmentation stages**

Expression of zRhoA remains ubiquitous until 24 hours post-fertilisation (hpf) (fig. 3.3f,g). However, levels of expression are not uniform within the embryo - by the 8 somite stage, expression levels are slightly higher in the developing CNS and the presumptive notochord than in the formed somites and presomitic mesoderm. By 24hpf, the major area of expression is the anterior CNS, encompassing the eyes and brain. There is also a weak upregulation of expression in anterior regions of the newly-formed notochord and anterior mesendoderm (fig. 3.5a,b) - levels in the somites and posterior body are visible but declining.
Fig. 3.2 ClustalW alignment of zRhoA peptide sequence against RhoA proteins from other species

At the amino acid level, zRhoA is highly conserved to other known RhoA proteins, including the invertebrate Aplysia homologue. Areas necessary for correct functioning are indicated, 1-5.

* indicates a single, fully conserved residue
: indicates that a 'strong' group is fully conserved
. indicates that a 'weaker' group is fully conserved

(see Appendix 2 for definition)
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3. Cloning and Expression of zRhoA and zRac1

Fig. 3.3 The expression of zRhoA during zebrafish embryogenesis

a) 4-cell stage, antisense probe
b) early-mid-blastula, antisense probe
c) 50% epiboly, antisense probe
d) tailbud, antisense probe
e) tailbud, sense probe
f) 7 somites, flatmounted, antisense probe
g) 24hpf, antisense probe
h) 36hpf, antisense probe
i) 48hpf, antisense probe
j) 72hpf, antisense probe
k) 24hpf, sense probe
l) 72hpf, sense probe

Embryos were probed with antisense RNA from the zRhoA clone, except for e, k and l which were probed with sense RNA from the same clone as a control. Expression is ubiquitous until 24hpf, after which time it becomes restricted to the head and notochord. Arrowheads in h, i, j denote the caudal extent of expression in the brain.

bl blastoderm br presumptive brain em embryonic tissue nc presumptive notochord psm presomitic mesoderm s formed somites y yolk

hpf - hours post fertilisation

Scale bars - 100μm, f) 50μm
3. Cloning and Expression of zRhoA and zRac1

3.2.2.3 Expression during pharyngula and hatching-period stages

By 36hpf, the expression of zRhoA is similar to that at 24hpf, however there has ceased to be expression in the developing musculature of the trunk and tail (fig. 3.3h). Expression in the notochord is stronger and extends along its full anterior-posterior extent (fig. 3.5c,d), and the expression in the mesendoderm is also maintained, in the putative gut precursors.

At 48hpf, the beginning of the hatching period, the same expression pattern is seen in the notochord and mesendoderm (fig. 3.4a,c) with additional weak staining in the neural tube (fig. 3.5e,f). Looking anteriorly, there is widespread expression in the head region, with clear staining in the hindbrain interneurons and in the emergent pectoral fin buds (fig. 3.4b).

By 72hpf, the latest larval stage tested, the transcript has ceased to be present in the fin buds (fig. 3.4e), notochord and mesendoderm (fig. 3.4f; fig. 3.5g,h). There has also been a refinement of expression anteriorly, such that the ubiquitous expression in the eye has become restricted to the retinal ganglion cell layer and the optic nerve (fig. 3.4e). Expression is also high in the developing jaw (fig. 3.4e).

Hybridisation of embryos at tailbud, 24hpf and 72hpf with a sense zRhoA probe as a negative control show no hybridisation (fig. 3.3e,k,l) suggesting that the antisense staining patterns seen result from true interactions between the labelled antisense probe and the endogenous sense mRNA.
Fig. 3.4 Closer examination at later stages of development reveals more clearly defined domains of zRhoA expression

a) 48hpf, lateral view
b) 48hpf, dorsal flatmount
c) 48hpf, lateral flatmount
d) 72hpf, lateral view
e) 72hpf, ventral flatmount
f) 72hpf, lateral flatmount

Viewing at an increased magnification reveals subtleties in the zRhoA expression pattern. At 48hpf, there is distinct staining in rows of hindbrain interneurons (white asterisks, b) and in the emergent fin buds (fb). Looking laterally, there is clearly staining in the notochord (nc) and the mesendoderm (black asterisks, c). By 72hpf, there is a decrease in expression at most of these sites - however, there appears to be a condensation of the generalised expression in the eye to the retinal ganglion cell layer and the optic nerve (rgc, on e) and increased expression in the developing jaw (j).

fb fin buds j developing jaw no notochord on optic nerve rgc retinal ganglion cell layer

Scale bars - a), d) 100µm; b), c), e), f) 50µm
Fig. 3.5 At progressive timepoints during development, \textit{zRhoA} is expressed in different regions of the trunk

a) 24hpf  
b) trunk vibrotome section, marked in a)  
c) 36hpf  
d) trunk optical section, marked in b)  
e) 48hpf  
f) trunk cryosection marked in e)  
g) 72hpf  
h) trunk cryosection marked in g)  

Arrowheads indicate the position of the notochord. Expression is upregulated here between 24hpf and 36hpf but has been extinguished by 72hpf. White asterisks indicate regions of staining in putative mesendoderm (b, d, f). There is also feint staining in the neural tube (\textit{nt}) - both of these regions have been downregulated by 72hpf. 

\textit{nt} neural tube.  

Scale bars - a), c), e), g) 100\(\mu\)m; b), d), f), h) 25\(\mu\)m
3.2.3 Cloning of a partial zebrafish zRacl fragment

3.2.3.1 Isolation of a zRacl clone

A slightly different approach was taken in the cloning of the zebrafish Rac1 homologue. Degenerate primers designed to flank a 300bp conserved region of the Rac gene (gift; Kate Nobes, LMCB; see Appendix 3 for sequences) were used in the PCR amplification of an embryonic zebrafish phagemid library (constructed by Anukampa Barth, Zebrafish Group, UCL.) These fragments were subcloned and twenty-four were sequenced, of which nine were random DNA sequence and fifteen were fragments of zebrafish Rac1 with varying degrees of homology. Clone 13 was found to be the most homologous and so was used to screen a zebrafish late-somitogenesis stage λZAP library as before. Again, after three rounds of screening, twenty-seven positive clones were isolated and four of the fourteen clones found to be positive after Southern blotting were sent away for automated sequencing. After sequence comparison using the BLAST database, it was seen that all four clones contained a Rac1 insert - however, all were found to be lacking sequence at either the 5' or 3' termini. Clone 23y was found to have the greatest degree of homology to other, known Rac1 sequences and so further characterisation was carried out with this clone, which was known from there on as zRacl.

The 1.5kbp insert was analysed using the Gene Jockey software package and the open reading frames and hence amino acid sequence deduced. The clone was found to consist of a 546bp partial coding sequence (see Appendix 1) followed by around 1kbp of 3' UTR - this would give rise to a putative protein lacking the first ten N-terminal amino acids (fig. 3.6).

3.2.3.2 Evolutionary comparisons with Racs from other species

The zebrafish zRacl protein contains the expected conserved motifs required for GTP-binding and GTpase activity (marked 1 to 4, fig. 3.6), as well as the C-terminal C-A-A-X post-translational isoprenylation site required for correct membrane targeting. Using the ClustalW program, the deduced amino acid sequence from zRacl was aligned with sequences of assumed homologues in other species (fig. 3.7). As with the zRhoA protein, the zRacl fragment also showed a considerable degree of conservation, being, for instance, 98% identical to the human and Xenopus sequences and 89% identical to the Drosophila sequence. Likewise, there was between 70 and 78% conservation at the DNA level.
Fig. 3.6 **Nucleotide sequence and deduced peptide sequence for the zRac1 coding region**

The deduced coding sequence contains an open reading frame lacking the initial 10 amino acids of the Rac1 protein. The remainder of the sequence is complete, including the 3' stop codon at nucleotide position 577. The sequence is numbered relative to the start of the deduced coding sequence.
D G A V G K T C L L I S
31 GAC GGG GCT GTG GGA AAA ACA TGC CTT CTG ATC AGC
Y T T N A F P G E Y I P
67 TAC ACC ACC AAT GCT TTT CCT GGG GAA TAC ATG GTC
T V F D N Y S A N V M V
103 ACT GTG TTT GAT AAT TAT TCT GCA AAT GTG ATG GTC
D G K P V N L G L W D T
139 GAT GGG AAA CCA GTA AAC CTG GGA TTG TGG GAT ACA
A G Q E D Y D R L R P L
175 GCA GGA CAA GAG GAT TAT GAC AGG CTT CGA CCG CTT
S Y P Q T D V F L I C F
211 TCC TAC CCT CAG ACG GAT GTG TTC CTG ATC TGC TTC
S L V S P A S F E N V R
247 TCT CTT GTG AGT CCG GCC TCA TTT GAA AAC GTT CGT
A K W Y P E V R H H C P
283 GCA AAG TGG TAT CCG GAG GTC AGA CAT CAT TGC CCT
N T S I I L V G T K L D
319 AAC ACT TCA ATA ATT CTT GGG ACT AAG CTT GAT
L R D D K D T I E K L K
355 TTG CGA GAT GAC AAG GAC ACC ATC GAA AAG TTA AAG
E K K L T P I T Y P Q G
391 GAG AAG AAA CTT ACT CCC ATC ACC TAC CCT NAA GCC
L A M A K E I G A V K Y
427 CTT GCT ATG GCC AAA GAA ATA GGA GCT GTG AAG TAC
L E C S A L T Q R G L K
463 CTG GAG TGC TCT GCC TTA ACC CAA CGC GCC CTT AAA
T V F D E A I R A V L C
499 ACT GTG TTT GAT GAA GCC ATC CGT GCA GTG CTG TGT
P P P V K R R R R R C L
535 CCA CCT CCG GTG AAG AGG AGG AGA AGA AGA GT T C TC
L L *
571 CTT CTG TGA
3. Cloning and Expression of zRhoA and zRac1

Fig. 3.7 ClustalW alignment of zRac1 peptide sequence against Rac1 sequences from other species

The zebrafish protein is highly conserved with respect to sequences from both close and more distant evolutionary homologues. Areas required for GTP binding and GTPase activity are labelled 1-4.

* indicates a single, fully conserved residue
: indicates that a ‘strong’ group is fully conserved
. indicates that a ‘weaker’ group is fully conserved

(see Appendix 2 for definition)
3. Cloning and Expression of zRhoA and zRacl

3.2.4 Expression of zRacl during development

3.2.4.1 Expression during early embryogenesis

To determine the expression pattern of zRacl during embryogenesis, in situ hybridisation was carried out at a variety of developmental stages. Up to and including tailbud stage, expression was found to be ubiquitous (fig. 3.8a-d) - again, the presence of the zRacl transcript prior to the switching to zygotic transcription at mid-blastula transition (fig. 3.8a,b) implies a maternal contribution of zRacl messenger RNA to the early embryo. Sections through embryos at the 50% epiboly stage show that the transcript is present in both the epiblast and involuting hypoblast (data not shown). No signal is seen using the sense RNA probe as a control, suggesting that the ubiquitous expression seen is a real pattern and not merely background staining.

3.2.4.2 Expression during segmentation stages

Expression of zRacl remains ubiquitous throughout somitogenesis (fig. 3.8f), with levels appearing more uniform than at the corresponding stages for zRhoA expression (compare fig. 3.3f with 3.8f). By 24hpf, expression is still widespread but becoming weaker in the trunk and tail compared with the high levels in the head (fig. 3.8g).

3.2.4.3 Expression during pharyngula and hatching-period stages

A broadly similar expression pattern is seen for zRacl as is found for zRhoA. At 36hpf, there is a high level of expression in the developing eyes and brain (fig. 3.8g) and weaker but ubiquitous expression throughout regions posterior to this. A few regions stand out as having upregulated the transcript at this point in development - throughout the length of the body, the notochord (fig. 3.10b, c) is stained, as is ventral mesoderm in the trunk (fig. 3.10b). Travelling anterior to posterior down the embryo, midway down the body there are lateral patches of stained cells adjacent to the horizontal myoseptum of the somites (fig. 3.10b) which probably correspond to nascent fast muscle fibres (Devoto et al., 1996) and continuing posteriorly, there is strong staining in the urogenital pore (fig. 3.10b).
3. Cloning and Expression of $zRhoA$ and $zRac1$

Fig. 3.8 The expression of $zRac1$ during zebrafish embryogenesis

a) 4-cell stage, antisense probe
b) early-mid-blastula, antisense probe
c) 50% epiboly, antisense probe
d) tailbud, antisense probe
e) tailbud, sense probe
f) 7 somites, flatmounted, antisense probe
g) 24hpf, antisense probe
h) 36hpf, antisense probe
i) 48hpf, antisense probe
j) 72hpf, antisense probe
k) 24hpf, sense probe
l) 72hpf, sense probe

Embryos were probed with antisense RNA from the $zRac1$ clone, except for e, k and l which were probed with sense RNA from the same clone as a control. Expression is ubiquitous until 24hpf, after which time it becomes restricted to the head, notochord and putative mesendodermal structures. Arrowheads in h, i, j denote the caudal extent of expression in the brain.

**Abbreviations:**
- **bl:** blastoderm
- **br:** presumptive brain
- **em:** embryonic tissue
- **nc:** presumptive notochord
- **psm:** presomitic mesoderm
- **s:** formed somites
- **y:** yolk

**hpf:** hours post fertilisation

Scale bars - 100µm, f) 50µm
3. Cloning and Expression of \( z\text{RhoA} \) and \( z\text{Rac1} \)

Fig. 3.9 Subtleties in the \( z\text{Rac1} \) expression pattern become apparent later in development

a) 48hpf, lateral view  

b) 48hpf, dorsal flatmount  

c) 48hpf, lateral flatmount  

d) 72hpf, lateral view  

e) 72hpf, ventral flatmount  

f) 72hpf, lateral flatmount

By 48hpf, the widespread expression of \( z\text{Rac1} \) seen earlier in development has become restricted. Anteriorly, there is an upregulation of expression in the diencephalon (\( \text{di} \)) and in the developing jaw rudiment (\( \text{j} \)). There are expression domains in the developing fin buds (\( \text{fb} \), b) and the notochord (\( \text{nc} \)), both of which are downregulated by 72hpf (e, f respectively). White asterisks in c denote expression in the viscera, and at both 48hpf and 72hpf, there is strong expression in the uro-genital opening (black asterisks, c, f).

\( \text{di} \) diencephalon  \( \text{fb} \) fin buds  \( \text{j} \) jaw  \( \text{no} \) notochord  \( \text{on} \) optic nerve  \( \text{rgc} \) retinal ganglion cell layer

Scale bars - a), d) 100\( \mu \text{m} \); b), c), e), f) 50\( \mu \text{m} \)
Fig. 3.10 At progressive timepoints during development, \textit{zRac1} is expressed in different regions of the trunk

a) 36hpf
b) trunk cryosection, marked in a)
c) tail, oblique cryosection marked in a)
d) 48hpf
e) trunk vibrotome section, marked in d)
f) tail optical section, marked in d)
g) 72hpf
h) trunk cryosection marked in g)

\textit{zRac1} is expressed in the notochord (\textit{nc}) at 36hpf and 48hpf, but by 72hpf, this expression has diminished. There is also a considerable level of staining in the viscera prior to 72hpf (black asterisks, b, c, e) and in the nascent fast muscle fibres (\textit{X}) and the urogenital pore (black asterisk, c) at 36hpf. There is little, if any, staining in the neural tube (\textit{nt}) beyond 36hpf.

\textit{nc} notochord, \textit{nt} neural tube.

Scale bars - a), d), g) 100\mu m; b), c), e), f), h) 25\mu m
3. Cloning and Expression of \( z\text{RhoA} \) and \( z\text{Racl} \)

By 48hpf (fig. 3.8i), the expression pattern has changed very little - there is still strong expression in the notochord (fig. 3.9c; fig. 3.10e, f) and ventromedial mesendodermal structures (fig. 3.9c; fig. 3.10f) which are thought to be precursors of the gut and excretory systems. There are additional, ventrolateral patches of stained cells in the trunk (fig. 3.10e) which probably demarcate emergent blood vessels. Looking anteriorly, there is \( z\text{Racl} \) expression in the nascent pectoral fin buds and there is a refinement of the expression domains in the head, with clear restriction occurring in the diencephalon and the eye (fig. 3.9b).

By the 72hpf, protruding mouth stage, expression in regions posterior to the brain is all but extinguished (fig. 3.9f; fig. 3.10e, f) and the transient expression in the fin buds has also been downregulated (compare figs. 3.9b and e). There is strong expression in the forming jaw (fig. 3.9e) and expression in the eye is clearly restricted to the retinal ganglion cell layer and the optic nerve.

Again, hybridisation with the sense RNA probe at 24hpf and 72hpf (fig. 3.8k,l) showed no staining, suggesting that the antisense staining seen is a result of true hybridisation between antisense probe and endogenous mRNA.

3.2.5 Northern blot analysis

To confirm the results seen by in situ hybridisation, total RNA was extracted from embryos at developmental stages corresponding to those used for the in situ - 50% epiboly, 6-10 somites, 24hpf, 48hpf and 72hpf. These latter two stages were separated into head and trunk/tail portions prior to RNA extraction, to see if a difference in expression levels between anterior and posterior fractions could be discerned. The amount of total RNA present was measured by spectrophotometry and the samples loaded to give 1\( \mu \)g per lane - at a rough estimate of 1% of total RNA being mRNA transcripts, this would give around 10ng of mRNA per lane. In addition, three control lanes were loaded per gel, consisting of 100ng each \( z\text{RhoA} \), \( z\text{Racl} \) and \( \beta\text{-actin} \) sense RNA. As a control for the amount of RNA loaded on the gel for each stage, an additional blot was carried out and probed for the ubiquitous cytoskeletal \( \beta\text{-actin} \) transcript - this was found to be present at a uniform level at all of the given stages (data not shown). However, for all the blots probed, there was considerable cross-reaction between the sense control lanes and the antisense probe used (fig. 3.11Ab, c; fig. 3.12Ab, c). This was due to the control lanes being greatly enriched with respect to the amount of sense RNA present in each lane compared with the embryonic extracts.
3. Cloning and Expression of \textit{zRhoA} and \textit{zRacL}

Fig. 3.11 Total RNA extraction and Northern blot analysis for \textit{zRhoA} at different developmental stages

A Total RNA extractions. 1\mu g loaded per lane unless stated otherwise

\textbf{a} RNA molecular weight markers
\textbf{b} \textit{zRhoA} RNA (positive control), 100ng
\textbf{c} \textit{zRacL} RNA (negative control), 100ng
\textbf{d} \textit{\beta-actin} RNA (loading control), 100ng
\textbf{e} total RNA, 50\% epiboly embryos
\textbf{f} total RNA, 6-10 somite embryos
\textbf{g} total RNA, 24hpf embryos
\textbf{h} total RNA from head only, 48hpf embryos
\textbf{i} total RNA from trunk and tail, 48hpf embryos
\textbf{j} total RNA from head only, 72hpf larvae
\textbf{k} total RNA from trunk and tail, 72hpf larvae

The position of the 28S and 18S ribosomal RNAs is marked.

B Northern blot
Lanes loaded as above.
There is considerable background hybridisation in all of the three control lanes. However, there is clearly a consistent band of staining ca. 1.38kb in size for stages up to and including 48hpf, with there being a decrease in expression levels in the 48hpf tail, as predicted by the \textit{in situ} data (compare levels in lane \textbf{h} with lane \textbf{i}). Interestingly, there appears to be an alternative transcript being expressed in both the head and the tail at 72hpf at moderate levels - this RNA species was not identified in whole-mount \textit{in situ} nor in sectioned tissue.
Fig. 3.12 Total RNA extraction and Northern blot analysis for zRac1 at different developmental stages

A Total RNA extractions. 1µg loaded per lane unless stated otherwise

- RNA molecular weight markers
- zRhoA RNA (positive control), 100ng
- zRac1 RNA (negative control), 100ng
- β-actin RNA (loading control), 100ng
- Total RNA, 50% epiboly embryos
- Total RNA, 6-10 somite embryos
- Total RNA, 24hpf embryos
- Total RNA from head only, 48hpf embryos
- Total RNA from trunk and tail, 48hpf embryos
- Total RNA from head only, 72hpf larvae
- Total RNA from trunk and tail, 72hpf larvae

The position of the 28S and 18S ribosomal RNAs is marked.

B Northern blot
Lanes loaded as above.

Again, there is considerable background hybridisation in all of the three control lanes due to overloading of these RNAs. However, there is a clear, consistent band of 2kb present in all the stages tested. This too is reduced in 48hpf and 72hpf posterior portions compared to head segments (compare levels in lanes h and j with i and k respectively.)
3.2.5.1 zRhoA expression

Similar levels of expression (judged by staining intensity) are seen (fig. 3.11) for the 50% epiboly, 6-10 somites and 24hpf stages. At 48hpf though, there is a clear difference in the expression levels between the head and tail fractions (compare lanes h and i), there being less of the transcript present in the posterior portion of the embryo compared to the anterior. However, an interesting result is seen when the two portions of the 72hpf embryo (lanes j and k) are considered. In both lanes, there is the presence of a strongly-stained band of a slightly smaller size than bands in all the other embryonic lanes, being 1.1kbp as opposed to 1.38kbp. An explanation for this could be a splice variant of the transcript being present, or the switching to a different zRhoA isoform. This conflicts somewhat with the in situ data - it is possible that fluctuations in the hybridisation stringency during the processing of the Northern blot have allowed cross-hybridisation to take place. It is not possible to tell whether the 72hpf head portion, (lane j), also contains the 1.38kbp band on account of the spreading and intensity of the smaller band and indeed, to know which of these species is being revealed in the in situ studies.

3.2.5.2 zRacl expression

The expression profile for zRacl seen on the blot (fig. 3.12) follows more closely the pattern seen by in situ analysis. At the stages up to and including 24hpf, expression levels are more or less uniform. At 48hpf and 72hpf, the results mirror those seen by in situ, such that there is a higher level of the transcript in the anterior versus the posterior portions of the embryo (compare lanes h and j with i and k respectively.) The transcript seen is about 2kbp long.

3.2.6 Expression of zRhoA and zRacl in adult zebrafish brain

3.2.6.1 zRhoA expression

zRhoA is selectively expressed at all rostrocaudal levels in the adult brain (fig. 3.13d, e, f). In the forebrain, of which a caudal section is shown, there is expression around the edges of the tectum and in the central accessory pretectal nucleus and the dorsal zone of the periventricular hypothalamus. Moving more posteriorly, there are again clear regions of staining in the midbrain (fig. 3.13e), in particular the periventricular grey zones of the optic tectum and the valvula cerebelli. The torus longitudinalis and the corpus mamillare are also strongly stained. Finally, the bulk of the body of the cerebellum stains positive for zRhoA, as
3. Cloning and Expression of zRhoA and zRac1

Fig. 3.13 Adult brain sections reveal different expression patterns for zRhoA and zRac1

a), d), g), j) sections from areas of the forebrain
b), e), h), k) sections from areas of the midbrain
c), f), i), m) sections from areas of the hindbrain

a), b), c) Haematoxylin staining
d), e), f) in situ hybridisation using zRhoA antisense probe
g), h), i) in situ hybridisation using zRac1 antisense probe
j), k) in situ hybridisation using zRac1 sense probe
l) in situ hybridisation using zRhoA sense probe

APN accessory pretectal nucleus CC crista cerebellaris CCe corpus cerebelli CM corpus mammillare DI lateral zone of dorsal telencephalic area ENd dorsal part of entopeduncular nucleus GC griseum centrale Hd dorsal zone of periventricular hypothalamus HV ventral zone of periventricular hypothalamus IMRF intermediate reticular formation IR inferior raphe LVII lobus facialis NXm vagal motor nucleus PGm medial preglomerular nucleus PGZ periventricular grey zone PGZVal periventricular grey zone of valvula cerebelli PTN T1 torus longitudinalis V1Is sensory root of the facial nerve Vd dorsal nucleus of ventral telencephalic area Vv ventral nucleus of ventral telencephalic area
3. Cloning and Expression of \(zRhoA\) and \(zRacl\)

does the crista cerebellaris adjacent to the rhombencephalic ventricle. At the level pictured (fig. 3.13f), there is also clear staining in the sensory root of the facial nerve and the intermediate reticular formation. It could be argued that the staining seen results from 'trapping' of the probe in ventricular spaces and loosely packed areas of tissue - however, in situ hybridisation using a sense \(zRhoA\) probe (fig. 3.13l) gave a negative result, suggesting that this is not the case.

3.2.6.2 \(zRacl\) expression

Expression of \(zRacl\) is remarkably similar to \(zRhoA\) in adult brain structures (fig. 3.13g, h, i) Again, it is the ventricular zones (e.g. periventricular grey zone of the tectum, fig. 3.13h) and the nuclei (e.g. vagal motor nucleus, fig. 3.13i) which are positive by in situ hybridisation. Again, hybridisation with a sense probe failed to give a signal (fig. 3.13j, k).

3.2.6.3 Haematoxylin staining

To try to identify the composition of the areas expressing the \(zRhoA\) and \(zRacl\) transcripts, adult brain sections were treated with haematoxylin, an histological stain which marks the presence of nuclei. The results of this staining (fig. 3.13a, b, c) show the existence of densely packed nuclei, in areas such as the torus longitudinalis and the periventricular grey zones. It would appear that all the areas expressing \(zRhoA\) and \(zRacl\) are those enriched in nuclei.

3.3 Discussion

3.3.1 Rho GTPase family homologues in the zebrafish

Prior to this study, only one zebrafish small GTPase of the Ras superfamily had been reported in the literature - a ubiquitously expressed Ran homologue (Liao et al., 1997) - and no members of the Rho family had yet been described. The results presented in this chapter describe the cloning and developmental expression patterns of two members of the Rho family of small GTPases in the zebrafish. Two members of the family were identified after the screening of a late somitogenesis stage cDNA library - however, as the in situ data has shown them to be widely expressed at robust levels particularly during the first 24 hours of development, it is perhaps of little surprise that these genes in particular were pulled out from the screen, and in several separate clones at that. It seems that in order to isolate the more esoteric and specifically expressed family members, such as the homologues of the neural crest-
marking RhoB (Liu & Jessell, 1998) or the neural-specific cRaclb in the chick, a different approach to the screening would need to be taken. One method would be to generate PCR fragments corresponding to the more divergent, C-terminal regions of the sequences and to use these to screen the libraries, or to use one of the increasingly popular expressed sequence tags (ESTs) to screen for a full-length clone. Alternatively, the library construction itself could be targeted, making new libraries that are narrower and later in their time window than the one screened (e.g. 72hpf tail fragments) or that are generated from single organs or tissues (e.g. adult retina) and thus may be enriched for alternative Rho family members.

The number of possible Rho family members present in the zebrafish is another issue which needs to be addressed. So far, there are ten known members of the family in mammals, some with multiple isoforms, namely Rho (A, B, C isoforms), Rac (1, 2, 3 isoforms), Cdc42 (Cdc42Hs, G25K isoforms), Rnd1, Rnd2, Rnd3, RhoE, RhoD, RhoG, TC10 and TTF (Bishop and Hall 2000), but little, if anything, has been reported regarding their evolutionary relationship to each other. It is known that during the course of vertebrate evolution, several genome duplications occurred, the evidence of which can be seen through the presence of multiple gene clusters, such as the four Hox gene clusters present in tetrapod vertebrates (Amores et al., 1998). However, recent evidence suggests an additional genome duplication in the cyprinid lineage, giving rise to an additional set of genes and a 'pseudo-tetraploid' state in the zebrafish - again, using the Hox genes as an example, at least seven clusters have been identified (Postlethwait et al., 1998; Prince et al., 1998). The number of copies, if applicable, of zRhoA and zRac1 could be resolved by Southern blotting of genomic DNA, and also by genetic mapping - by determining the genetic map location of these genes, known duplicate areas could be screened for the presence of the same or divergent copies. It would also be interesting to see if these genes map to the same loci as any known mutants, which would give more information as to their function during embryogenesis and to what extent there may be redundancy.

3.3.2 Evolutionary relationships between zebrafish Rho GTPases and those from other species

3.3.2.1 zRhoA

There can be little doubt that the zRhoA clone is a true homologue of the RhoA genes found in other species. It shows an high degree of homology, at both the nucleotide and amino acid levels, to RhoA sequences cloned in even evolutionarily divergent species such as Aplysia (Anderson & Lacal, 1987). It could be argued that
the zebrafish clone is actually a RhoB or a RhoC homologue - however, comparing the amino acid sequences found for these proteins in the chick and the human (Malosio et al., 1997) would imply this to be unlikely - RhoB is highly divergent in its C-terminal 16 amino acids compared to RhoA, possessing a three amino acid insert adjacent to the C-A-A-X motif; conversely, RhoC lacks the first nineteen amino acids at the 5' terminus. Additionally, the widespread expression pattern in the zebrafish embryo adds weight to the clone's identification as a RhoA homologue - in other organisms examined such as Drosophila and the chick, a similar widespread pattern has been reported (Hariharan et al., 1995; Liu & Jessell, 1998) whereas, in the chick at least, RhoB is expressed more specifically and transiently in the dorsal neural tube and migrating neural crest (Liu & Jessell, 1998).

3.3.2.2 zRac1

A little more persuasion is needed to make the case for the second clone being a zebrafish Rac1 homologue. Along the length of the fragment present, there is clear homology at both the amino acid level (fig. 3.7) and at the nucleotide level, with an average identity of 93% and 76% respectively for the species considered, from mammals such as the cow to invertebrates such as Drosophila. Since the presumed first ten amino acids are missing, it could be argued that in the native protein, these are wholly divergent and this is a novel family member. I would counter this claim with the evidence from other species that Rac1 (both a and b forms in the chick) and Rac2 genes are identical across this region and that the variation which differentiates between them occurs primarily in the C-terminal half of the protein (Hariharan et al., 1995). Likewise, the clone is highly unlikely to be a homologue of the Drosophila DRac3 gene, since this is in the region of being only 50% identical to Rac1 at the amino acid level, and lacks amino acids 121-129 when compared to the human sequence (Sasamura et al., 1997). Additional data, again from the widespread expression of the transcript during embryogenesis, would point to this being Rac1 rather than the more neural specific Rac1b (Malosio et al., 1997) or Rac2, which is thought to be restricted to haemopoietic and thymopoietic lineages in some organisms (Williams et al., 2000).
3.3.3 Comparison of expression patterns in the zebrafish with those in other species

3.3.3.1 Expression of RhoA

As seen from the in situ and the Northern blotting data, \( zRhoA \) has a widespread and abundant expression profile during zebrafish embryogenesis. From very early cleavage stages, a strong signal is present implying a strong maternal contribution of \( zRhoA \) transcripts to the egg. It is not possible to determine the longevity of this messenger RNA, but the strength and ubiquity of expression at stages after mid-blastula transition and the initiation of zygotic transcription suggests that there is also a high degree of zygotic transcription. When the function of RhoA is considered, it is hardly surprising that such high levels of the transcript and, presumably, protein are found during the first 24 hours of embryogenesis - cell shape changes and morphogenetic tissue movements driven by the actin cytoskeleton are rife, as is signalling through various cascades triggered by Rho family activity (Fanto et al., 2000). So, the apparent downregulation that is seen at later stages provides somewhat of a puzzle - one would assume that structures in the embryo, such as the somites, which are still involved in active growth and morphogenesis at 72hpf would require the functions of RhoA. The in situ data would imply that this is not the case, as no transcript can seen in the trunk of the larva. I propose three possible explanations for this - firstly, there could well be mRNA present, albeit at 'housekeeping' levels too low to be identified by the in situ protocol. Alternatively, there may be switching to an alternatively-processed transcript which cannot be hybridised to by the antisense probe used during the protocol. Finally, it may well be that \( zRhoA \) expression is switched off and its function is taken over by other, redundant members of the Rho GTPase family. Evidence from the Northern blot analysis would add weight to the second hypothesis, but that is not to dismiss the other suggestions out of hand.

It is perhaps in the invertebrates where the most rigorous characterisation of RhoA's expression profile has been carried out. Several groups describe the cloning and expression in \( Drosophila \) (Hariharan et al., 1995; Sasamura et al., 1997) and \( C. elegans \) (Chen & Lim, 1994). The results shown lead to similar conclusions in both organisms, such that there is widespread expression during embryogenesis, with levels of the transcript being relatively uniform. However, looking more closely at \( C. elegans \), two salient points become apparent: firstly, although the level of the \( CeRhoA \) transcript peaks at embryogenesis and then decreases through the larval stages, examination of the protein levels present using a CeRhoA antibody shows a
peak at larval stages which decreases into adulthood, suggesting that some form of post-transcriptional or post-translational modification is occurring - this cannot be tested in the zebrafish until a reliable RhoA antiserum is obtained. Secondly, the CeRhoA protein is enriched above the general levels in the pharyngeal nerve ring, the co-ordination centre which receives projections from most of the animal's 302 neurons (Settleman, 1999) and in the anterior organ at the tip of the head, which contains chemo- and mechanosensory neurons. No expression is seen in the motor neurons or the interneurons, suggesting that CeRhoA has a specific role in receiving and co-ordinating external stimuli.

The RhoA expression profile in higher organisms tells a slightly different story. In the chick, expression is seen to be homogeneously expressed throughout the embryo at stage 6.5, including parts of the nervous system such as the tectum and the spinal cord (Malosio et al., 1997). This homogeneity of expression is seen at stage 8.5 in the cerebellum and retina, but in the spinal cord, expression is found to be concentrated in the ventricular zone and the ventral region in the vicinity of the nascent motor neurons. Looking more closely at this region, a rostro-caudal dynamic of expression was seen at stage 10 (Liu & Jessell, 1998), in that ubiquitous expression was found at neural plate and neural fold stages but in regions which had undergone neural tube closure, the levels of RNA were decreasing in the ventral neural tube and later, in the cells at the dorsal midline.

It might be expected that of the species studied, the expression pattern of the Xenopus XRhoA transcript might be the closest to that seen in the zebrafish. It would appear that this is not necessarily the case, although it might be true at earlier embryonic stages (Wunnenberg-Stapleton et al., 1999). In gastrula stage embryos, XRhoA is expressed in the ectoderm and the involuted mesoderm corresponding to the organiser region, as well as the endoderm - this domain of expression could not be ascertained by in situ hybridisation, but appeared to be present on analysis by RT-PCR. A similar profile was seen at the mid-neurula stage but by the tailbud stage, transcripts were found to be enriched in the mesoderm and ectoderm of the head and tail.

To summarise, no direct comparisons nor firm conclusions can be drawn from the developmental expression patterns of zebrafish zRhoA and its homologues in other species. The ubiquity of expression is seen in many organisms and reflects a requirement for these GTPase molecular switches in processes requiring actin-based cell shape changes and movements, such as gastrulation and elongation of the embryo. Broadly speaking though, there are parallels between species, such as
obvious requirements for RhoA in all the embryo types studied, and an upregulation in developing neural tissues. However, more and detailed analyses need to be performed to verify whether the tissue-restriction seen, for example, in the notochord occurs in other vertebrates. Likewise, it seems unlikely that zRhoA expression is extinguished wholly in structures such as the somites during any stage of development as Rho is thought to function in 'housekeeping', particularly in cells that are actively involved in morphogenesis.

3.3.3.2 Expression of zRacl

As for zRhoA, there are abundant maternal and early zygotic levels of the zRacl transcript, which persist ubiquitously until 24hpf. After this time however, a similar restriction begins to be apparent, with the upshot being strong expression levels in the developing brain and eyes and in axial mesodermal and visceral structures such as the notochord and urogenital pore, respectively. These data are concomitant with a requirement for zRacl in cell movement and the cytoskeletal rearrangements required for the axonal projections as well as for the establishment of epithelial polarity - this latter may well explain the persistence of transcript levels in the developing mesendodermal structures, since the gut is lined with polarised epithelial cells. Again though, the same questions arise regarding the restriction and lack of staining within the trunk at later stages - however, a clearer answer may be given by the Northern blot data which imply the presence, albeit at low levels, of transcript in regions which are negative by in situ.

In Drosophila, Rac1 is seen to have strong and ubiquitous expression in the precellular and later, cellular blastoderm, with there being a maternal contribution to the presence of the transcript prior to the initiation of zygotic transcription (Luo et al., 1994). After gastrulation, levels of Rac1 become enriched in the developing CNS and the mesoderm - by stage 13 and beyond, expression remains high in the gut but decreases in the somatic mesoderm. Luo et al (1994) propose that Rac1 has an important role in the processes of axonogenesis and myoblast fusion during muscle formation. Coupled with the preponderance of expression in the visceral mesoderm, this is very similar to the expression pattern described here for the zebrafish zRacl homologue.

Other than in Drosophila, there has been little detailed expression analysis for Rac1. In C. elegans, the presence of two Rac1 transcript species has been reported (Chen et al., 1993) but both were found in whole animal extracts, with no whole-mount in situ information being given. In the chick (Malosio et al., 1997),
cRaclA is described as being ubiquitous throughout the developing embryo, being homogeneously expressed. A more detailed analysis is carried out in the neural retina - two transcripts of sizes 2.4kbp and 0.95kbp are found, with the former, major species remaining at a constant level between the stages of E6 and E12, whereas the latter gradually weakens. This corresponds to a window in embryonic development when the retinal ganglion cells are extending processes and pathfinding within the tectum - the expression of zRacl seen in the retinal layer and optic nerve at 48hpf could well be analogous to the similar expression seen in the developing chick. As for RhoA though, the implication is that Rac1 is required ubiquitously in cells at early stages of embryogenesis for processes again involving cell movements and tissue morphogenesis.

3.3.4 Northern blot analysis

As seen from Northern blots performed for zRhoA and zRacl, both genes are expressed strongly during early embryogenesis and at 48hpf, are more weakly expressed in the posterior regions compared to the anterior. However, the expression profiles diverge at 72hpf, wherein there appears an additional, smaller zRhoA RNA species which is expressed strongly in both the anterior and posterior fragments. Comparing these results seen with Northern data from other organisms, these results are neither surprising nor conclusive. Different species, it would appear, may express one or more than one RhoA and Rac1 during development, judged by the number of bands seen to hybridise. For example, C. elegans and the marine ray Discopyge ommata both express a single RhoA transcript of 2kbp and 2.4kbp respectively (Chen & Lim, 1994; Ngsee et al., 1991) whereas two major species of transcript, 1.8kbp and 1.45kbp, are found in the developing chick retina (Malosio et al., 1997), of which the smaller transcript is found to be downregulated earlier than the larger (although expression of both is weak by E12). Similarly, there are variations in the sizes and numbers of Rac1 transcripts present depending on the organism studied - a 1.7kbp and a 0.9kbp-sized transcript are found in C. elegans embryonic and early larval stages, neither of which are present in the adult (Chen et al., 1993) whereas the 2.4kbp and 1.1kbp Rac1 species were found in all the human cells tested, ranging from placental to brain tissue, with the smaller transcript being less abundant than the larger (Didsbury et al., 1989). Hence, I propose that the Northern blot data presented here may be used to support the gross assertion that RhoA and Rac1 are expressed in a spatio-temporal manner, but little can be deduced from the size and number of RNA species present on account of the varied data found from other organisms.
3.3.5 Rho family expression in developing and mature brain tissue

The function of the Rho family of GTPases in developing and developed brain tissue has been speculated to involve the remodelling of the synapses, the basis of plasticity and learning (Nakayama et al., 2000; Threadgill et al., 1997). RhoA has been shown *in vitro* to bring about neurite retraction in NIE-115 and PC12 cells (Kranenburg et al., 1999; Nishiki et al., 1990) whereas Rac1 has been seen to be involved in growth cone formation and pathfinding, and neurite extension both *in vitro* and *in vivo*. In the adult rat brain, RhoA and Rac1 were seen to have identical but specific expression patterns (Olenik et al., 1997) with transcripts being found in the hippocampus, thalamus, piriform cortex, cerebral neocortex and, particularly abundantly, the cerebellum. It is difficult to speculate on what the analogous structures to many of these may be in the zebrafish, but in the rat cerebellum, high levels of RhoA and Rac1 are seen in a cellular distribution typical for that of neurons with a strong signal in the Purkinje cells - a similar distribution is seen in the zebrafish cerebellum (fig. 3.13f). It is interesting to note that N-chimaerin, a molecule which activates GTPases and induces lamellipodia and filopodia to form, is also expressed at its highest levels in the hippocampus, cortex and cerebellum of the adult rat brain (Kozma et al., 1996; Lim & Michael, 1992) concomitant with the GTPases' role in bringing about the neural shape changes required for new connections to be made. It is highly likely that a similar situation is found in the adult zebrafish brain, with the RhoA and Rac1 transcripts having very similar expression patterns.

3.4 Conclusions

*zRhoA* and *zRacl* are highly conserved compared to their homologues in other species and are widely and abundantly expressed during zebrafish embryogenesis. Their sites of expression in the embryo and adult brain are in cells which are presumed to require rapid and efficient remodelling of the actin cytoskeleton in order to bring about morphogenetic processes such as gastrulation and axonal outgrowth.
CHAPTER FOUR

An Investigation into the Role of the RhoA GTPase During Zebrafish Embryogenesis

4.1 Introduction

The Rho family of GTPases have been well characterised with respect to their capacity to bring about cell shape changes, via downstream rearrangements of the actin cytoskeleton (Hall, 1998). The ability of cells to alter their morphology and to locomote is central to the processes underlying tissue movements during embryonic morphogenesis, and thus it is unsurprising that Rho GTPases have been shown to play a key role in such processes, both in vitro and in vivo (Harden et al., 1999; Ridley & Hall, 1992).

The zebrafish embryo provides a superb model for the observation of the cellular movements involved in processes such as epiboly and gastrulation and so it was decided to inject synthetic, capped RNAs into 1-8 cell stage embryos and examine the resultant effects on development (Holder & Xu, 1999). Differing forms of human RhoA were used - the wild-type, G14Rho; dominant negative, N19Rho, which is thought to act by remaining in the GDP-bound state and sequestering guanine nucleotide exchange factors (Ridley & Hall, 1992); and constitutively active V14Rho, which is refractory to the actions of GTPase activating proteins in the cell and also has a reduced intrinsic GTPase activity, and thus remains in an active conformation (Hall, 1998). Previous studies utilising similar, human gene constructs have indicated that these proteins can disrupt the endogenous Rho pathways in a species as divergent as Drosophila (Harden et al., 1995; Strutt et al., 1997), suggesting a high degree of functional conservation during evolution. In addition to the human Rho constructs, RNA encoding the C3 exoenzyme was also injected. This is a protein identified from Clostridium botulinum which covalently modifies all forms of Rho in the cell and thus blocks their function (Ridley & Hall, 1992) - it was proposed that the phenotype thus generated may complement that shown by the injected dominant negative RhoA.

All constructs mentioned were kind gifts from Alan Hall and Kate Nobes, LMCB, London.
4. The Role of the RhoA GTPase During Zebrafish Embryogenesis

4.2 Results

4.2.1 Synthetic RNA is translated efficiently in vivo

The original, donated clones were in the pRK5 vector and as such, did not contain any of the sites required for *in vitro* RNA synthesis and only possessed a single Myc epitope tagging the Rho/C3 insert. Initial results, in which these constructs were injected into early embryos as DNA, proved to be unsatisfactory - the embryos displayed non-specific symptoms of toxicity rather than any defined phenotype and the single Myc tag proved to be insufficient to be recognised by the anti-Myc antibody, assuming that the DNA was being expressed at all. Hence, a different approach was pursued. Using a PCR-based approach, the inserts were subcloned into pCS2+MT, a construct based on the Bluescript vector with the addition, of SP6 and T7 promoters and an SV40 polyA site for *in vitro* RNA synthesis; together with six Myc epitope tags, which were positioned at the 5' end of the subcloned inserts. These constructs were generated for all three forms of the human Rho gene and for the C3 exoenzyme, and capped RNA was synthesised from them *in vitro* (Krieg & Melton, 1984). These RNAs were injected into one cell of 1-8 cell stage embryos at a starting concentration of 100ng/µl and then titred (down, in most cases) accordingly once it had been established that expression of the RNA was occurring *in vivo*. Injected embryos were allowed to develop at 31°C until they had reached the 50% epiboly stage, whereupon they were fixed and immunostained for the Myc epitope (fig. 4.1) - all three of the Rho constructs were expressed efficiently, as seen by the mosaic pattern of cells staining positively (arrowheads, fig. 4.1b, d, f). However, no staining was seen in the C3 injected embryos - the severity of the phenotype (see below) would suggest that the RNA was being expressed but that the protein was present in amounts below the threshold of sensitivity for the antibody. The embryo pictured in fig. 4.1g had been injected with 10ng/µl C3 RNA, as a concentration higher than this was found to result in rapid death before the embryos could be fixed and analysed meaningfully. Hence, 10ng/µl was the concentration used for subsequent analysis of C3 RNA injections.

4.2.2 Overexpression of wild-type or dominant negative Rho has no effect on embryogenesis

Embryos injected with either the G14Rho (wild-type) or the N19Rho (dominant negative) RNA showed no discernible phenotype over the range of concentrations tested. For the G14Rho construct, a total of 222 embryos were injected with 25ng/µl to 100ng/µl and in all cases, the embryos appeared to be
Fig. 4.1 Staining for the Myc epitope shows the expression of RNA injected at the 1-4 cell stage in the mid-gastrulation embryo

a), b) G14Rho injected, 100ng/μl

b), d) N19 Rho injected, 100ng/μl

c), e) V14Rho injected, 50ng/μl

g) C3 RNA injected, 10ng/μl

h) uninjected control embryos

Arrowheads in b, d, f indicate cells expressing the myc-tagged RNA. As expected, expression is mosaic within the embryo. Little, if any, staining can be seen in the C3 injected embryo - this is probably due to the minimal amounts of the tagged enzyme which are present. No staining is seen in the uninjected controls.

Scale bars - a), c), e), h) 100μm; b), d), f) 50μm
4. The Role of the RhoA GTPase During Zebrafish Embryogenesis

normal at 50% epiboly, 24hpf and 48hpf despite immunostaining at these first two stages revealing a significant number of cells expressing the Myc epitope and, it was assumed, the Rho fused to it. Toxicity levels were not determined for this construct. Similar results were obtained for the dominant negative construct - N19Rho RNA was injected at concentrations from 40ng/µl to 200ng/µl (n=359 in total) and no specific phenotype was seen, aside from the generalised toxicity phenotypes seen in ~19% of those injected with the highest concentration (x=20 from sample size n=117). For both of these RNAs, three batches were synthesised independently and were seen to be of the expected size when electrophoresed and viewed using ethidium bromide and UV light - however, no batch was found to give a specific phenotype when injected. Additionally, an RNA encoding green fluorescent protein was synthesised in parallel from the pCS2-eGFP vector and this was found to be efficiently translated when co-injected with the G14Rho RNA, as deemed by the presence of fluorescently-labelled cells within the injected embryos at 70% epiboly and 24hpf (data not shown).

4.2.3 Injection of constitutively active V14RhoA

4.2.3.1 Injection of constitutively active V14RhoA produces an epiboly phenotype that is dose-dependent

When embryos were injected with RNA encoding the constitutively active form of RhoA, a novel phenotype was seen a matter of hours after injection, whereby the cells of the blastula cap were unevenly distributed across the dome of the yolk cell, giving a pointed or 'quiffed' appearance (data not shown; fig. 4.2 shows a later resolution of these effects). This phenotype was seen with the initial RNA concentration of 100ng/µl and was found to be dosage-dependent in its effects on the injected embryos over the range of concentrations assayed, in terms of the numbers of embryos displaying the phenotype and the severity of the effect. The graph below shows the concentration injected plotted against the percentage of abnormal embryos generated.
4. The Role of the RhoA GTPase During Zebrafish Embryogenesis

Even though the background, toxicity phenotype was not seen at any of the concentrations assayed, it was decided to carry out further injections using the RNA at 50ng/μl, since this produced a phenotype in nearly 80% of injected embryos and greatly reduced any likelihood of RNA toxicity.

4.2.3.2 *V14Rho* injected embryos are abnormal during epiboly and have a shortened axis

Injection of *V14Rho* at 50ng/μl produces an epiboly phenotype which can vary in severity within the same clutch of injected embryos (fig. 4.2). Two to three hours after injection at the 1-4 cell stage, the embryos appear 'lopsided', with cells amassing asymmetrically on the yolk and in more severe cases, the animal cap becoming conical. By the time the uninjected sibs have reached 50% epiboly, there is a clear thickening of the blastoderm and the epiboly front has failed to migrate as far vegetally as it ought. This phenotype varies across a spectrum of severity (fig. 4.2b through e): some only have a thickening of the blastoderm to a lesser or greater extent (fig. 4.2b, c), whereas in others, there is a constriction around the circumference of the blastoderm, deforming the underlying yolk and giving the embryo 'baked bean'-shaped (fig. 4.2d). In the most severely affected embryos, there is a composite of these phenotypes, giving rise to a very distorted embryo (fig. 4.2e).
Fig. 4.2 *V14Rho* RNA expression produces a variable epiboly phenotype

a) uninjected embryo, 50% epiboly
b), c), d), e) 50ng/μl *V14Rho* RNA injected embryos
f) uninjected embryo, tailbud stage
g), h) 50ng/μl *V14Rho* RNA injected embryo

The constitutively active *V14Rho* RNA produces a consistent yet variable phenotype during gastrulation and epiboly, as shown by the gradation in severity from b to e. The range of phenotypes seen is from a thickening of the blastoderm (arrowheads, a, b) to deformation of the entire embryo (e).

By the tailbud stage, these phenotypes are resolved as a shortening of the embryonic axis (compare positions of the tailbud, tb, in f-h) and a thickening of the anterior extension (asterisks, f-h) - h represents a more severe injected phenotype than g.

**tb** tailbud

Scale bars - 100μm throughout
This early phenotype is resolved by tailbud stage (around 10 hours after injection with embryos incubated at 31°C) into a shortening of the embryonic axis and a shift of the tailbud itself towards the animal pole (fig. 4.2g, h) compared to the wild-type. Again, this reduction in axis length varies in severity, corresponding to the deformation seen at earlier stages (compare fig. 4.2g, h). The animal pole, however, remains more or less normally positioned, but there is a thickening of the anterior extension in the injected embryos compared to the wild-type.

4.2.3.3 *Injected embryos show errant expression of anterior and axial markers*

Injected embryos at the tailbud stage were fixed and assayed by *in situ* hybridisation for the expression of markers specific for anterior and axial tissues. *shh* labels the nascent axial mesoderm (Krauss et al., 1993), which at the tailbud stage is present in a column running from anterior to posterior in the midline of the embryo. Its anterior extent abuts the edge of the neural plate, the anterior limit of which is labelled with *emxl* (Morita et al., 1995) (fig. 4.3). In injected embryos, the morphology of the neural plate only deviates mildly from the horse-shoe shaped structure seen in the wild-type, even in the embryos severely affected with respect to other features (fig. 4.3a, b, c, d). However, the expression of *shh* reveals defects in the axis of the embryo that do vary in severity - some embryos are only mildly affected, with the *shh* domain resembling that seen in the wild-type (fig. 4.3b) or having fluctuations in its breadth (fig. 4.3c); whereas others display a curvature in the anterio-posterior extension of the domain, with the anterior extent being off-centre in its contact with the *emxl* domain (fig. 4.3d).

*nfl* marks the cells fated to become the notochord (Schulte-Merker et al., 1992; Schulte-Merker et al., 1994) and this too displays a curved expression pattern along the midline of the injected embryos compared to the wild-type (fig. 4.3e, f) - however, the expression does appear to extend as far anteriorly as in the sib.

The most anterior mesoderm is correctly positioned but expanded in the injected embryos examined - *hgg1* (A. Barth, unpublished) marks the presumptive hatching gland, anterior to the prechordal plate. Cells expressing this gene are present as in the wild-type, but the domain of expression is broader in the mediolateral direction.
Fig. 4.3 Expression of axial and anterior markers at tailbud stage in V14Rho RNA injected embryos

a), e) uninjected sib
b), c), d), f) V14Rho RNA injected

Expression of the anterior marker enxl shows that the morphology of the anterior neural plate is slightly affected in V14Rho RNA injected embryos. However, the axial mesodermal marker shh shows a gradation in phenotypic severity, from wild-type (b) through slightly broadened to show a curved deviation in its anterior-posterior direction in severe cases (c). This curved deviation is also demonstrated by the expression of ntl in flatmounted embryos (e, f). There is also an expansion of the hatching gland marker hgg1 in the injected embryos.

Scale bar - 50μm throughout
4. The Role of the RhoA GTPase During Zebrafish Embryogenesis

4.2.3.4 Early brain specification is altered in injected embryos

The transcription factor pax2.1 (Krauss et al., 1991) marks the presumptive midbrain-hindbrain boundary at the tailbud stage, and so can be used as a marker for early patterning defects in the embryo. The gene is expressed in two oblique, symmetrical stripes meeting at the midline in the rostral third of the embryo, and shows a marked disruption in its expression domains in VMRho RNA injected embryos. For instance, in the VMRho injected embryo shown in fig. 4.4, there is a clear compaction of the domain when viewed laterally as compared to the wild-type (fig. 4.4a, b). This corresponds to a reduction and an asymmetry of expression in the dorsal aspect, the stripes appearing to have failed to converge adequately towards the midline.

4.2.3.5 Posterior mesoderm is positioned incorrectly in injected embryos

By the end of epiboly, the posterior mesodermal cells have migrated to the vegetal half of the embryo and circumvent the tailbud. Several genes are expressed in these cells, which are fated to populate the paraxial mesoderm and later, the somitic musculature. For example, papc (Yamamoto et al., 1998), the paraxial protocadherin, is involved in specifying paraxial versus axial mesoderm; her1 (Muller et al., 1996), is a homologue of the Drosophila pair-rule gene hairy and is expressed in dynamic stripes later corresponding to the primordia of alternating somites; whereas MyoD (Weinberg et al., 1996) is expressed in adaxial cells flanking the presumptive notochord at the tailbud stage.

Expression of all three of these markers is disrupted in the injected embryos (fig. 4.5). papc expression fails to surround the tailbud and the stripes of expression have lost their symmetry across the midline (fig. 4.5b). Likewise, the expression domain of her1 appears to be encroaching into the tailbud region and the stripes of expression are greatly disturbed (fig. 4.5d) - again, there is a loss of symmetry in the two lateral halves of the embryo. Finally, MyoD has failed to extend anteriorly (fig. 4.5f) and the two stripes are irregular. The expression of all the markers shown has broadened laterally around the circumference of the yolk, suggesting that there has been a failure in the migration of cells to the vegetal half of the embryo and in the convergence of these cells to the dorsal side of the embryo during gastrulation and epiboly.
The domains of \textit{pax2} expression are altered in \textit{V14Rho} injected embryos. For example, from a lateral aspect, (white asterisks, a, b) the domain appears shortened in the anterior-posterior direction. Looking dorsally, there is a clear asymmetry in the expression domains in the injected embryo, with one of the domains becoming narrower across the medio-lateral axis (arrowheads, d).

Scale bars - 50\textmu m throughout
Fig. 4.5 Expression of posterior mesoderm markers in V14Rho RNA injected embryos

a), c), e) uninjected sibs, tailbud stage
b), d), f) 50 ng/μl V14Rho RNA injected embryos, tailbud stage

a), b) papc expression
c), d) herl expression
e), f) MyoD expression

Expression of all three posterior mesodermal markers is disrupted in the V14Rho RNA injected embryos. papc fails to circumscribe the tailbud, and the paraxial stripes (white arrowheads, a, b) become broader with irregular boundaries in the injected embryo. Similarly, herl’s stripes are disrupted (white asterisks, c, d) but there appears to be an encroachment of the expression flanking the tailbud into the tailbud itself. MyoD’s stripes, marking the presumptive adaxial cells, are also uneven and fail to project as posteriorly as the in the uninjected sib (arrowheads, e, f).

Scale bar - 50μm throughout
4.2.3.6 **VI4Rho injected embryos have anterior patterning defects later in development**

Injected embryos and their uninjected sibs were incubated at 31°C until they had almost completed epiboly and then were incubated at 22°C overnight to allow them to be inspected at mid-somitogenesis stages the next morning. The injected embryos displayed a striking brain and eye phenotype (fig. 4.6a-f), such that their brains had 'hairpin bends' and there was varying degrees of eye fusion. In some cases, the axis was also shortened in the anterior-posterior aspect - this was not seen in the uninjected sibs, nor in any embryos incubated at higher temperatures, and thus suggests a temperature-dependent component to the phenotype. The same anterior phenotype was seen after overnight incubation at 28°C or 31°C (fig. 4.6h) and in all cases, there was a variation in severity in the symptoms seen, for instance in the extent of eye reduction and/or fusion present - however, this did not correlate with the strength of phenotype seen during epiboly and gastrulation stages. Nor were there any effects apparent on mesodermal derivatives, as all of the injected embryos, both with and without anterior defects, developed wild-type notochordal and somitic tissue.

4.2.3.7 **The morphological defects are mirrored by disrupted gene expression patterns in the brain and eye**

At later stages, *pax2.1* expression is maintained in the caudal portion of the midbrain and is also activated in the pronephric ducts, the otic vesicles and the optic stalks (Krauss et al., 1991). In the *VI4Rho* injected embryos examined, all retain the expression domain in the midbrain (fig. 4.7b, c, e) but there was an alteration in its position relative to the anterior of the embryo, apparently shifting rostrally - in some cases slightly (fig. 4.7b), in others more severely (fig. 4.7c). There is also a reduction of expression in the otic vesicle, and in some embryos this domain is lacking entirely (fig. 4.7e). Expression of the transcription factor *pax6.1* (Macdonald et al., 1994) in the hindbrain may show a slight expansion in the dorso-ventral aspect (fig. 4.8b). This is a subtle effect, however. The spacing between this domain and the anterior expression domain in the forebrain remains remarkably similar to that seen in the wild-type (compare fig. 4.8a, b; 4.8c, d).

It is in the eye where there are strikingly aberrances in the expression domains of these two transcription factors. *pax2.1* marks the presumptive optic stalk cells, whereas *pax6.1* marks the cells destined to form the retinae. Injected embryos have a marked reduction and in some cases, almost an absence of *pax2* expression.
4. The Role of the RhoA GTPase During Zebrafish Embryogenesis

Fig. 4.6 Injection of 50ng/μl V14Rho RNA has a severe affect on the patterning of the anterior region of the embryo

a), b) uninjected sib, 12 somites

C), d) V14Rho RNA injected embryo, 15 somites

e), f) V14Rho RNA injected embryo, 16 somites

g) uninjected sib, 21 somites

h) V14Rho RNA injected embryo, 21 somites

a), c), e) lateral views

b), d), f), g), h) dorsal views

Embryos a)-f) were incubated at 22°C, whereas those in g), h) were incubated at 28°C.

In the injected embryos, the notochord and somites form as normal, it is the most anterior structures that are affected. Arrowheads in d), f) and h) demarcate the varying degrees of ‘hairpin bend’ that can be seen in the injected specimens. Asterisks indicate the developing eye primordia, which are reduced and fused in the treated embryos. In addition, some of the injected embryos incubated at 22°C have a shortened rostro-caudal axis - this phenotype is not seen in the uninjected sibs nor in either group when incubated at 28°C.

Scale bars - 100μm throughout
Fig. 4.7 Expression of *pax2.1* in injected embryos at 18-20 somites

a), d) uninjected sibs
b), c), e) *VMRho* RNA injected

There is an obvious reduction in *pax2* expression in the developing optic stalk (white asterisks) in injected embryos - this can be seen from a lateral aspect (a-c). However, dorsal flatmounts show a fusion of the usually twinned domain in the most severely affected specimens (compare d with e). There is also a reduction in otic vesicle (ov) expression. The expression at the midbrain-hindbrain boundary remains wild-type in all cases, and can be used as a gross marker for the shortening of the anterior region of the brain from the rostral tip of the embryo to the midbrain-hindbrain boundary (m) in the injected embryos.

**m** midbrain-hindbrain boundary  **ov** otic vesicle

Scale bar - 100µm
Fig. 4.8 Expression of *pax6* at 18-20 somites

a), b) uninjected sibs  
c), d) 50ng/μl *V14Rho* RNA injected embryos

Expression of the transcription factor *pax6* is subtly altered in the injected embryos. There is an expansion of the expression domain into the ventral diencephalon (black asterisks) which can be seen clearly in both lateral- and dorsal-mounted embryos. There is also evidence of dysmorphogenesis in the hindbrain (black arrows), as shown by the altered shape of the domain in the *V14Rho* treated embryos.

Scale bars - 50μm throughout
4. The Role of the RhoA GTPase During Zebrafish Embryogenesis

when viewed laterally (fig. 4.7b, c). Looking dorsally, it becomes clear that these domains are not only reduced, but there is a fusion of the domains in the centre of the eye field (fig. 4.7e) implying that only a single, central optic stalk would have been formed. Concomitant with this, there is an expansion of the cells with retinal fates - these encroach into the floor of the ventral diencephalon (fig. 4.8b, d) in the injected embryos.

4.2.3.8 V14Rho generates patterning defects in the CNS

The eye phenotype seen at mid-somitogenesis stages becomes more obvious in older animals, having resolved into incomplete separation of the eyes (fig. 4.9b) or, in the most severe cases, complete cyclopia (fig. 4.9c). Immunostaining with the anti-acetylated tubulin antibody reveals an axon scaffold in which some structures are entirely absent (fig. 4.10). A typical, V14Rho injected embryo (48hpf) is seen to have a reduced tectum (fig. 4.10b). The tract of the posterior commissure is defasciculated (fig. 4.10d) and the tract of the post-optic commissure has failed to form. The optic nerve projects aberrantly (fig. 4.10f), but this is not surprising given the severity of the eye phenotype.

4.2.3.9 Injected embryos have cranio-facial defects

The injected embryos survive, hatch and are as motile as their wild-type sibs. However, their long-term survival would be in doubt on account of the cranio-facial defects that become apparent in later larval stages (fig. 4.9d, e, f, g). The lower jaw fails to reach the anterior extent of the embryo and projects ventrally, giving the embryo an 'open-mouth' appearance - this is unlikely to develop into a functioning structure meaning that the embryo would be unable to feed, leading to death. The embryo pictured has a milder form of eye fusion, but the eyes are clearly closer together than in the wild-type and turned in towards the midline.

4.2.3.10 V14Rho injection phenocopies the silberblick mutation

The mutation silberblick (slb) was identified during the Tübingen mutagenesis screen and was classed as a forebrain-patterning mutant, in which there was initially a delay in embryonic axis extension which resolved in an eye fusion phenotype (Heisenberg et al., 1996). A comparison of the morphology of these embryos with that of embryos injected with V14Rho reveals striking similarities between the two (fig. 4.11). At tailbud stage, both types of embryo have failed to extend fully from the animal to vegetal poles, though it must said that although
Later in embryogenesis, *V14Rho*’s effects on facial morphology become more pronounced

a) uninjected sib, 42hpf
b), c) *V14Rho* RNA, 50ng/μl injected embryos, 42hpf
d), f) uninjected sib, 80hpf
e), g) *V14Rho* injected embryo, 80hpf

Later in development, the *V14Rho* injected embryos exhibit an eye phenotype (arrowheads) from a mild fusion and downturn of the eye towards the midline (g) through eye fusion (b) to total cyclopia (c).

There is also a craniofacial phenotype, such that the lower jaw fails to migrate to its full anterior extent (compare asterisks in d, f to those in e, g) and appears to gape open.

Scale bars - a)-c) 100μm; d)-g) 50μm
Fig. 4.10 Staining for anti-acetylated tubulin at 48hpf reveals neural defects in embryos injected with 50 ng/μl V14Rho RNA

(a), (c), (e) uninjected wild-type sibs
(b), (d), (f) V14Rho RNA injected embryos

Lateral views - a, b; dorsal views - c, d; ventral views - e, f

In the V14Rho injected embryos, the axonal scaffold is, in the main, formed normally. However, there are a few exceptions - the tract of the post-optic commissure (a, b) is absent, and the optic nerve itself projects aberrantly (compare e with arrowheads in f). Additionally, the posterior commissure (c, d) appears to be defasciculated.

AC anterior commissure ON optic nerve TAC tract of the anterior commissure TecN tectal neuropile Tel telencephalon TPC tract of the posterior commissure TPOC tract of the post-optic commissure

Scale bars - 100μm throughout
Fig. 4.11 Homozygous slb embryos have a phenotype similar to V14Rho RNA injected embryos

a) wild-type, uninjected embryo, tailbud stage  
b) 50ng/μl V14Rho RNA injected embryo, tailbud stage  
c) slb homozygous embryo, 95% epiboly  
d) wild-type embryo 24hpf, lateral view  
e) slb embryo 24hpf, lateral view  
f) wild-type embryo 24hpf, dorsal view  
g) slb embryo 24hpf, dorsal view  
h) wild-type embryo, 80hpf  
i) 50ng/μl V14Rho RNA injected embryo, 80hpf  
j) slb homozygous embryo, 96hpf

By the end of epiboly, the similarity between the injected embryos and slb homozygotes is clear - both fail to extend anteriorly, and the tailbud is shifted away from the posterior pole, resulting in a shortened axis. (This is patent in the slb embryo despite being slightly temporally behind the other embryos shown).

By 24hpf, the slb embryos display a similar eye and brain phenotype to the V14Rho injected embryos - arrowheads in e, g indicate areas of eye fusion and black crosses (X) denote aberrant brain morphology - compare with Fig. 4.6.

At later stages of development, the jaw phenotype is remarkably similar (asterisks, h-j).

a anterior-most part of embryo  
b tailbud

Scale bars - a)-c) 100μm; d)-g) 50μm
being similarly affected at the vegetal pole, slb embryos are more severely affected in their failure to extend anteriorly. At 24hpf, slb have phenotypic elements resembling those of the less severe \textit{V14Rho} embryos, with a mild fusion of the eyes in the midline (fig. 4.11e, g) and aberrant morphology in brain. Finally, when the embryos are compared at later larval stages, they become almost indistinguishable (compare fig. 4.11i, j). Even though the \textit{V14Rho} injected embryo is not pigmented due to PTU treatment and a few hours younger than the \textit{slb} individual, the gaping jaw is present in both, as is the tilt of the eyes towards the midline.

4.2.4 \textbf{Injection of C3 exoenzyme RNA}

4.2.4.1 \textit{C3 RNA is functional at very low doses}

The initial concentration of RNA injected, 100ng/\textmu l, was thought to be too high, in that embryos appeared to be sick within 2 hours of injection, with death ensuing over the subsequent few hours. It transpired though that this phenotype was seen at all the concentrations of RNA injected, even at levels as low as 1ng/\textmu l. Further experimental work was carried out injecting the RNA at 10ng/\textmu l, which allowed some embryos to survive to 8hpf at 28°C (6hpf at 31°C) but was unfortunately too low a concentration for the resultant protein to be visualised using the anti-Myc antibody in the embryos injected with this concentration - any increase in the amount of \textit{C3} RNA injected gave rise to embryos too fragile to be fixed and stained. However, there can be little doubt as to the functioning of the C3 protein which is synthesised, judging from the rapidity and severity of the phenotype produced.

4.2.4.2 \textit{C3 RNA produces a phenotype shortly after injection}

As mentioned above, the injection of the \textit{C3} RNA produced a severe phenotype within a couple of hours after injection (fig. 4.12). The embryos appeared to lose the cellularity of the blastula cap, accompanied by a loss of tension in the yolk cell, the surface of which would become pitted with cracks and dimples. In order to narrow down the time frame over which these processes were occurring, a timed series of photographs was taken at 10-20 minute intervals for a \textit{C3} injected embryo and an uninjected sibling (figs. 4.14, 4.13). This timecourse revealed that the exoenzyme begins to exert its action, blocking all the functioning Rho in the cell, within 25 minutes after being injected. The efficiency with which the RNA is translated into functional protein and the enzyme exerts its effects is stunning - an
Fig. 4.12 Injection of C3 RNA produces a phenotype within a few hours of injection

a) uninjected sib, t+3h
b) C3 RNA injected embryo, t+3h

Embryos injected with the C3 RNA construct have a pronounced phenotype within hours of injection. There is an apparent loss of cellularity and the yolk cell loses tension and forms cracks and dimples.

Scale bar - a), b) 100μm
Fig. 4.13 Time-lapse series showing development of a wild-type control embryo

t=n in each frame indicates the time elapsed in minutes since siblings were injected with RNA encoding the C3 exoenzyme. As can be seen, the control embryo develops normally with cell division occurring every 10-20 minutes.

Scale bar – 50µm
Fig. 4.14 Time-lapse series showing development of a C3 RNA injected embryo

t=n in each frame indicates the time elapsed in minutes since the embryo was injected with RNA encoding the C3 exoenzyme. The enzyme exerts its effect rapidly on the embryo – by t=24, there is already an effect on cell division, such that the embryo has one normal cleavage furrow (arrowhead) and one lacking (asterisk.) White arrowheads (t=114, t=184, t=254) indicate transient deformation occurring in the yolk cell, in the form of cracks and dimpling.

Scale bar – 50μm
hour after injection, the embryo is barely recognisable compared to its sib. After an hour and a half has elapsed, pits and fissures begin to appear on the yolk's surface - it would appear that these are transient structures, perhaps reflecting changes in tension within the embryo. Seven hours after injection, the C3-treated embryo is still alive, but the blastula cap has collapsed onto the yolk and there is no sign of any of the usual features of epiboly, such as the thickening of the blastoderm margin, as seen in the wild-type control.

4.2.4.3 C3 does not block gene transcription in injected embryos

C3 RNA injected embryos seem, from their morphological appearance, to be shutting down cellular functions. To see whether this was the case, injected embryos were taken through in situ hybridisation for the ubiquitously expressed zRacl and the zygotic, mesodermal marker goosecoid (gsc), which is expressed in cells which have involuted at the shield during early gastrulation (Schulte-Merker et al., 1994). The expression of zRacl is remarkably similar in the injected embryos compared to wild-type sibs, assayed 4h post-injection - there is ubiquitous expression at robust levels in both embryos (fig. 4.15a-d). Nevertheless, zRacl is a gene with a strong maternal contribution (see Chapter 3) and it is not possible to say whether synthesis of this gene has been triggered de novo at mid-blastula transition or if the mRNA being visualised is residual from the maternal component.

To try to differentiate between these possibilities, embryos were hybridised with a gsc probe to see if the C3 RNA injected embryos retained the capacity for zygotic transcription, and whether the 'cells' had developed any sense of identity with respect to being, in this case, axial mesoderm. In the wild-type, gsc was expressed as expected in the hypoblastic cells involuting at the shield; however in the C3 injected embryo, gsc was also expressed but in superficial, seemingly random patches on the surface of the embryo (fig. 4.15e, f).

Both the zRacl and gsc results showed though that the injected embryo does retain some aspects of cellularity and vestiges of an epiboly front (fig. 4.15c).

4.2.4.4. C3 injected embryos are capable of nuclear division

Phosphorylated histone 3 is a marker of dividing cells, being upregulated as the nuclei's chromatin begins to condense and decreasing again as the daughter nuclei mature. Thus, the antibody to this protein (D. Delaney, PhD Thesis 1999) is an effective marker for such dividing cells in the zebrafish embryo. Immunostaining
Fig. 4.15 Injection of C3 RNA has no effect on gene transcription per se

a), b) wild-type, 4h post-injection

c), d) 10 ng/μl C3 RNA injected, 4h post-injection

e) wild-type, 6h post-injection

f) 10 ng/μl C3 RNA injected, 6h post-injection

a), c), e) lateral views

b), d) animal views

f) oblique view

Embryos hybridised for the zRac1 RNA, a ubiquitously expressed gene, showed no alteration in the expression pattern of this transcript (c, d) compared to their non-injected sibs (a, b). However, the effects of the RNA on cell morphology are clearly visible - the black lines in a and c demarcate the epiboly front which is irregular in the injected embryos compared to their sibs. Also, the white arrowheads in d show areas with apparently lower levels of expression, which appear as ‘pits’ on the surface of the embryo.

Although injected embryos retain the ability to express zygotic genes, they cannot do so in a regional-specific manner. Goosecoid is a mesodermal marker expressed in cells which have involuted at the shield (e). Injected cells express this gene in random, superficial patches (arrowheads, f).

Scale bars - 100μm throughout
Fig. 4.16 Expression of P-H3, a marker of dividing cells, is downregulated in C3 RNA injected embryos

a), c), e) uninjected embryos, t+3h
c), d), f)-h) 10ng/μl C3 RNA injected embryos

Again, there are two phenotypes seen affecting cell division in C3 injected embryos. In the first case, the number of cells dividing appears to match that in the wild-type situation, with maybe even a slight increase. However, looking at the intensity of staining in the injected embryos would suggest a decrease in the level of phosphorylated histone present - chromatin condensing into individual chromosomes (asterisks, e) is not seen in these embryos.

The second phenotype seen is that where the nuclei have mostly ceased dividing (occasional scattered cells, g) - again, no condensed chromatin is visible (h). This embryo was probably very close to death.

Scale bars - a), b), g) 100μm; c), d) 50μm; e), f), h) 25μm
carried out on C3 RNA injected embryos and the sibs revealed two phenotypes with respect to cell division (fig. 4.16). In the first instance, the C3 injected embryos appear to be unaffected in levels of cell proliferation (fig. 4.16a, b). A closer inspection reveals that although this may be the case, the chromatin is less-darkly stained and actual chromosomes (fig. 4.16e) cannot be seen in the injected embryos. This could well be attributed to the failure of some aspect of the cytoskeleton in the nucleus, such as one that may be required for the assembly of condensed DNA onto the central protein scaffold (Stryer, 1995). Alternatively, the depletion in visible phosphorylated histone may reflect a block in a Rho-mediated kinase cascade. In any case, these factors do not affect the ability of the nuclei to divide, ultimately, nor affect the transcriptional machinery therein.

The second of the two phenotypes is an almost complete shutdown in nuclear division, whereby other C3 injected embryos lose an extensive proportion of labelled nuclei, retaining occasional clumps here and there (fig. 4.16g). It might well be that such embryos are ones that were closest to dying and were in the process of closing down their cellular machinery when fixed.

4.3 Discussion

The results presented in this chapter suggest an essential role for Rho GTPases during embryonic development. Expression of a constitutively active form of the RhoA gene induces cyclopia, implying incorrect migration of the prechordal plate and overlying neuroectoderm. The complete abrogation of Rho function brought about by the expression of a C3 exoenzyme construct has a severe effect on the embryo, leading to a failure in the normal pattern of cell division and migration and subsequent death.

4.3.1 Overexpression of wild-type or dominant negative RhoA has no effect on zebrafish embryogenesis

4.3.1.1 Injected zebrafish embryos are indistinguishable from wild-type sibs

I show that the overexpression of either wild-type human RhoA or, perhaps more surprisingly, its dominant negative counterpart has no effect on zebrafish embryogenesis. The embryos pass through epiboly and gastrulation, processes both thought to be heavily reliant on the correct and precise movement of the cells and remodelling of their underlying actin cytoskeleton, as normal. There are no effects on the later processes of segmentation, both in the somites of the trunk and the
regionalisation of the hindbrain, as seen by the regular, undisturbed morphology of both; nor are there any later effects on the patterning and outgrowth of the central nervous system - staining with the anti-tubulin antibody shows a normal axon scaffold with all projections reaching their correct targets. The injected embryos are, in all aspects considered, phenotypically wild-type. In support of these data, embryos treated with the Rho-kinase blocking agent, Y-27632, complete epiboly unperturbed (K. Woolley, unpublished data), implying at some level a redundancy of function for Rho in the early embryo.

4.3.1.2 *Overexpression of these constructs in Drosophila blocks cellularisation and dorsal closure*

These results conflict with data gleaned from similar experiments in other organisms. The majority of the work pursued in this area has been carried out in *Drosophila*, where differing effects are seen when Rho function is interfered with at differing stages of development. A dominant negative *DRholl* (the homologue of RhoA) construct expressed widely in the early embryo under the control of the *nanos* promoter produces obvious defects in gastrulation - there is a delay in the formation of the ventral furrow which subsequently fails to extend at its posterior end. The midgut fails to invaginate and germband extension also fails (Barrett et al., 1997). This lethal phenotype is remarkably similar to the phenotype generated by knocking out the function of the upstream activator, *DRhoGEFl*, or those phenotypes seen in the *folded gastrulation* (*fog*) and *concertina* (*cta*) mutants. *fog* is a secreted extracellular ligand, whereas *cta* is the Gα subunit of a heterotrimeric protein linked to the *fog* receptor. Hence, *DRhoGEFl* is postulated to act as the signalling link between receptors at the cell surface and the Rho cascade. Mutations at any point in this pathway cause defects in the cell shape changes required for gastrulation. Similar results are found in the examination of the role of RhoA in dorsal closure in *Drosophila*, where expression of an *N19RhoA* transgene has dramatic effects on the cytoskeleton of the leading edge cells (Harden et al., 1999), giving rise to a segmentally repeated loss of myosin and a loss of contraction of these cells. Embryos homozygous for a lethal P-element insertion abrogating Rho1 function display a phenotype in which dorsal closure does occur, but in a disorganised fashion - cells at the midline are inappropriately shaped, some being stretched, others pinched together. There is also a failure in head involution in these embryos, showing that Rho1 functioning is essential for *Drosophila* embryonic development (Magie et al., 1999).
Microinjection of protein reagents corresponding to the dominant negative forms of human RhoA or the C3 exoenzyme into the posterior poles of cellularizing Drosophila embryos gives a spatiotemporally specific analysis of Rho function, which is more finely-tuned than the widespread expression mentioned previously (Crawford et al., 1998). Within minutes of injection of either protein, there is a halt in the invagination of nascent cleavage furrows brought about by disruption of the cytoskeleton, with a breakdown of the regular, hexagonal actin array into aggregates. There is a progressive disorganisation of the nuclei, which fall away from the surface of the embryo as a presumed secondary consequence of the cortical actin breakdown. These effects are dose-dependent with respect to the amount of protein injected and can be ameliorated by pre-injection of the embryo with the wild-type or constitutively active versions of RhoA, neither of which generate a phenotype when injected alone - injected embryos pass through cellularisation and gastrulation as usual.

4.3.1.3 *Modifying expression of RhoA leads to epithelial polarity defects*

Restricted overexpression of a wild-type transgene in the Drosophila eye imaginal disc in the cells posterior to the morphogenetic furrow gives rise to a particular eye phenotype (Hariharan et al., 1995). In the presence of one copy of this transgene, the eye appears normal but will deform easily under mild pressure, and has an irregular shape and arrangement of rhabdomeres. When a double dose of the transgene is received, the eye becomes very rough and there is a considerable disruption of the ommatidial architecture - the ommatidial cytoskeleton is disorganised and the later differentiation of the photoreceptor and pigment cells fails to occur correctly. These results were taken to mean that an increase in Rho expression in cells, although not affecting the determination of cell fate, impedes the ability of the actin cytoskeleton to tolerate shape changes and causes cells to become abnormal in their morphology.

Elsewhere, it is reported that strong loss of function RhoA alleles present a tissue polarity phenotype similar to that of the frizzled (Fz) and dishevelled (dsh) mutants which are downstream components of the Wingless signalling pathway (Fanto et al., 2000). A gain of function in these genes can be dominantly suppressed by a reduction in the amount of functioning RhoA. RhoA is also required for the polarity and number of hairs present on the wing i.e. RhoA participates in signalling from membrane-bound receptors to the nucleus in order to generate polarity in epidermal structures (Strutt et al., 1997). This dynamic developmental system which
requires cells to move and rotate in a coordinated way is achieved by modification of the cytoskeleton by small GTPases accompanied by Fz-induced nuclear signalling.

4.3.1.4 There is no clear reason for the lack of phenotype seen in zebrafish

By reviewing the data provided from such experiments in Drosophila, it is clear that no homologous phenotypes are generated by over-expression of wild-type or dominant negative RhoA in the zebrafish embryo. Embryos do not lag behind or fail to complete gastrulation; nor do they exhibit any problems with epithelial patterning or muscle induction (see 1.1.6.1). These results are not concordant with those from other organisms and speculation might be made as to why this is so. There may be an inefficient processing of the injected RNA, albeit an unlikely suggestion in the light of the effects seen with the other injected constructs and the widespread expression of the Myc tag. It may transpire that there is such a high level of interacting factors present in the embryo that the supposed sequestering of exchange factors and blocking of endogenous function does not occur at the level of exogenous protein it is possible to generate through the technique of mRNA injection - a way to test this theory would be to inject the protein reagents directly into fertilised eggs. The lack of an obvious phenotype is unlikely to stem from the use of the human homologues of the genes, as these have been found to function in Drosophila and Xenopus. It may well be a combination of the specificity of the dominant negative RhoA and the redundancy of function of shared homologues in the zebrafish that brings about this apparent anomaly.

4.3.2 Expression of constitutively active RhoA causes fusion of the eyes and aberrant brain morphology

4.3.2.1 V14Rho brings about an obvious epiboly phenotype

The effects on epiboly caused by the expression of constitutively active human RhoA are unequivocal. The embryos appear to have cells of the same absolute size and number as their uninjected siblings, ruling out an effect of the protein on the progression of the cell cycle or cytokinesis. However, the morphology of the injected embryos (fig. 4.2) at mid-epiboly stages would surely suggest a departure from the norm in terms of cell adhesion, with the cells remaining piled asymmetrically, typically on the presumptive dorsal side. Even the most severely affected embryos recover from this early digression though, and the movements of convergence and extension occur, albeit with some delay, resulting in a shortened embryonic axis.
4. The Role of the RhoA GTPase During Zebrafish Embryogenesis

In spite of the deviations seen during epiboly, \textit{in situ} hybridisation for the expression of genes marking the different germ layers, e.g. \textit{shh} for axial mesoderm, \textit{emx1} for anterior neuroectoderm, reveals that the correct induction of each tissue type has occurred - even in the apparent morphological absence of the shield, the zebrafish organiser (Shih & Fraser, 1996), in embryos with the more pronounced epiboly phenotype. I would speculate that this implies the putative shield cells need not necessarily be located in an obvious, physical cluster in order to exert their organisational function - perhaps the close apposition they are held in due to the altered adhesive properties of the cells at that point is sufficient for induction of the germ layers.

There is, however, a patent loss of some convergent extension capacity, which is unlike that seen in other, classical convergent extension mutants such as \textit{knypek} and \textit{trilobite} (Solnica-Krezel et al., 1996). There is a curved deviation of the \textit{shh} expression domain, yet it still abuts the anterior edge of the neural plate, marked by \textit{emx1} - this is not the case in either of these two mutants (Marlow et al., 1998), in which the axial mesoderm fails to project as far anteriorly as it ought.

It is perhaps in the posterior mesoderm that the failure to converge is most clear. Expression of markers in this region illustrates a situation where cells have failed to move dorsally and intercalate, thus failing to elongate the embryonic axis to its full extent. There is an asymmetry in the patterns seen, perhaps reflecting a mosaicism in the inheritance and expression of the injected mRNA in different cells. Again though, it would seem that the numbers and general positioning of cells expressing a given marker does not differ from that in the wild-type, supporting the hypothesis that cell identity is maintained despite deviations in cell positioning. Remarkably, the posterior mesodermal tissue derivatives remain phenotypically wild-type in the majority of embryos when examined 14-18 hours after tailbud stage. For instance, the somites are patterned normally, and there is induction and differentiation of functioning muscle tissue. Thus, it appears that the injection phenotype is, in some respects, transient.

4.3.2.2 \textit{This phenotype is unlike that seen in other organisms}

Although there is little, if any, data that is directly comparable to the effects of V14Rho RNA injection on gastrulation, it is worthwhile considering that provided from eggs and embryos treated at very early stages. When \textit{Xenopus} oocytes are injected with V14Rho protein in the presence of progesterone, their morphology changes such that there is a redistribution of pigments at the animal pole, giving it a
spotted appearance (as opposed to the usual, single white patch at this position caused by germinal vesicle breakdown). There is also an increase in the rate at which progesterone-induced maturation occurs in the presence of V14Rho, with the conclusion being that Rho is required to regulate cytoskeletal organisation during this maturation of the oocyte from the first meiotic prophase to an arrest in metaphase II (Mohr et al., 1990).

Looking a little later, in the fertilised Xenopus egg, constitutively active RhoA is seen to block the first cleavage in a dose-dependent manner, with a similar phenotype seen at a five-fold higher concentration of exogenous wild-type Rho (Drechsel et al., 1997). Cytokinesis is initiated at the correct time and position in the animal hemisphere, and the division furrow propagates towards the vegetal pole, but it fails to ingress fully and the daughter cells are not separated. At higher doses, there are additional erroneous events such as the rotation of partially cleaved blastomeres and contraction of the pigmented cortex into a small clot. Both of these events result in the lysis of the embryo. The failure of the furrow to ingress may result from the global increase in cortical actin that is seen stiffening the cortex to the extent that it becomes refractory to deformation - this may well be analogous to the effects seen in the injected zebrafish embryos, in that a stiffening of the cell cortices slows epiboly and makes convergent extension slothful.

In the early mouse embryo, constitutively active Rho injected into one cell of a 4-cell conceptus produces morphological alterations in the injected cell (Clayton et al., 1999). A critical point in mouse embryonic development is the process of compaction, occurring at the 8-blastomere stage: there is a redistribution of cell surface and cytoskeletal components and an increase in intercellular adhesion, mediated by E-cadherin. This is the process responsible for the earliest polarity in the embryo, and is essential for later differentiation of the trophoblastic and pluriblastic lineages. In V14Rho injected cells, this polarity begins prematurely, such that the nucleus of the injected cell is displaced to the apical edge. There is elongation of the cell along the axis perpendicular to the intercellular contact points, and an increased fragility of these embryos suggests a decreased adhesion in the injected cells. These results do not tally with any of the phenotypic characteristics of the injected zebrafish embryos - there is, if anything, a proposed increase in cell adhesion in the injected cells, or if not, an increased adhesion in their non-injected counterparts; there is no defect in the generation of any aspect of embryonic polarity.

An explanation for the phenotypes seen in the injected embryos could be that during the normal course of epiboly, Rho is not in fact activated - the in situ data
shown in chapter 3 shows a high level of the transcript but provides no other evidence as to its translation, if any, into protein nor whether this putative protein is in an active state or sequestered in the cytoplasm with GDIs. Consequently, the phenotypes seen could result from the activation of Rho-mediated pathways that are normally quiescent. This would tally with the absence of effects seen when G14Rho or N19Rho are injected, since they have no active pathways to target.

4.3.2.3 At later stages VMRho injected embryos have fused eyes and aberrant brain morphology

By mid-somitogenesis stages, the expression of VMRho has had an obvious effect on the patterning and formation of the brain and eyes, derivatives of the anterior neural plate. This could have been prefigured at tailbud stage by the disrupted expression of the brain patterning marker pax2 and the slightly offset morphology of the anterior edge of the neural plate compared to the wild-type, although the strength of the resultant phenotype is surprising given the degree to which the posterior mesoderm was seen to be affected and subsequently recovered. Perhaps even more surprising is the apparent abutment of the axial mesoderm and the neural plate - yet, the degree of eye fusion and cyclopia generated is that usually seen when the two are far distant and shh signals from the midline fail to repress the expression of pax2 in the neural plate. Thus, the midline cells ectopically express pax2 and differentiate as optic stalk at the expense of retina (Macdonald et al., 1995) and there is a deficit in ventral CNS tissue in this region. In the injected embryos, there is a midline fusion of the pax2 domains despite shh signalling being correctly positioned in the anterior-posterior direction at the tailbud stage.

Additionally, there is a reduction in fore- and mid-brain structures in the injected embryos when visualised with the anti-acetylated tubulin antibody - this could be a secondary deficit brought about by the cell migration defects. Accompanying this is the apparent defasciculation of the posterior commissure and the absence of the tract of the post-optic commissure. These effects could result from a primary deficit brought about by the constitutively active Rho disrupting neural process outgrowth - for instance, blocking Rho activity in vitro has been shown to stimulate axon outgrowth even on inhibitory substrates (Lehmann et al., 1999) so it may be that constitutive activation has the opposite effect in the injected embryos. Indeed, Rac1 has been identified as a playing a key role in Drosophila axon guidance (Luo et al., 1994), so it is possible that crosstalk with V14Rho interferes with its function in nascent axon tracts. Alternatively, the axon tract loss might be a secondary effect caused by the failure of proper tissue movements and thus an
4. The Role of the RhoA GTPase During Zebrafish Embryogenesis

incorrect positioning of the requisite axon guidance signals: the dynamic interactions between the growth cone and underlying attractive/repulsive cues are vital for the correct targeting of neurons (Suter & Forscher, 1998; Wahl et al., 2000). There is even a possibility that the axon tract phenotype results from the misspecification of brain tissue into tissue of another identity, thus disallowing the formation of neurons - however, this is unlikely when the amount of tissue present in the embryonic head is considered: the V14Rho injected embryos are generally smaller than their wild-type sibs implying a reduction rather than a respecification.

There is evidence from experiments carried out in Xenopus that RhoA is required for the induction of head structures (Wunnenberg-Stapleton et al., 1999), such that the co-injection of XRhoA RNA in its native or V14 form together with a dominant negative BMP receptor can induce the development of ectopic head structures on the ventral side of the embryo. Conversely, dominant negative XRhoA can reduce the ectopic heads induced by the ventral injection of cerberus. It is possible that the effects seen in the injected zebrafish embryos result from the participation of RhoA in a head-induction pathway, but this would not account for the epiboly and convergence phenotypes also seen. It is, however, perfectly possible that the effects seen are the consequence of the Rho's participation in many and varied pathways during embryogenesis.

4.3.2.4 Cyclopia is generated by incomplete partitioning of the eye field, as seen in the silberblick mutant

During eye development and morphogenesis, an initial single field of progenitors in the antero-medial region of the neural plate is split bilaterally by the anterior progress of midline tissue in the neural plate which elongates during convergent extension (Woo & Fraser, 1995). This physical bisection of the initial eye field is not sufficient for eye formation - there is a requirement for signals from the axial mesendoderm. Surgical removal of portions of the prechordal plate in Amblystoma (Adelmann, 1930) results in a cyclopic phenotype, and the spontaneously-occurring cyclopic talpid mutant in the chick is known to have aberrant prechordal plate morphogenesis and a displacement of the optic stalks towards the midline (Ede, 1964). Signals from the ventral midline of the CNS must also be correctly positioned for the specification of retina and optic stalk cell fates (Ekker et al., 1995; Macdonald et al., 1995).

Two classes of mutants displaying fused eyes are seen in the zebrafish. The first, including mutants such as oneeyedpinhead (oep) and cyclops (cyc) (Brand et
4. The Role of the RhoA GTPase During Zebrafish Embryogenesis

al., 1996; Hammerschmidt et al., 1996; Hatta et al., 1991), are lacking portions of the axial mesendoderm and ventral CNS, resulting in fused retinal in the expense of a decrease in optic stalk tissue. In the second category, including the mutants silberblick (slb), knypek (kny), and trilobite (tri) (Hammerschmidt et al., 1996; Heisenberg et al., 1996; Solnica-Krezel et al., 1996), the morphogenesis of the axial mesendoderm and the overlying ventral CNS is disturbed, giving rise to a compression rather than a lack of midline tissue. This often results in varying degrees of incomplete eye separation, with an anterior fusion of the optic stalks. From the results seen in the VMRho injected embryos, the phenotype resembles more this second class of mutants, in particular the silberblick locus, since slb embryos have a normal body length whereas knypek and trilobite are somewhat shorter.

silberblick embryos are characterised at tailbud stage by their shorter, broadened axis and a narrow, elongated polster. The posterior prechordal plate and the notochord are also shortened and broader, indicative of a general migratory defect in the axial mesendodermal cells. At later stages, varying degrees of eye fusion are seen and it is interesting to note that while the tailbud phenotype is completely penetrant, the later eye phenotype is not seen in all the mutant embryos, some of them appearing to be wild-type (Heisenberg & Nusslein-Volhard, 1997). This is reminiscent of the VMRho injection phenotype - not all of those affected, even severely, during epiboly will develop eye and CNS defects. However, the eye phenotype in slb is thought to result from the shortened axial midline, whereby shh signalling falls short of its target cells in the anterior neural plate and pax2 is induced ectopically in medial regions of the neural plate. This is puzzling when considered in the context of the V14Rho injected embryos - at tailbud stages, the axial midline is clearly of wild-type length, but later embryos have a midline fusion of pax2 expression. This could be explained by convergence extension movements occurring between these two stages, which alter the relationship between the anterior axial mesendoderm and the neural plate.

Silberblick is thought to be required for the cell movements needed in both the cell intercalations of convergent extension and the anterior migration of axial mesendoderm cells, both of which contribute to the slb phenotype; whereas the convergence of the dorsal and paraxial mesoderm, as seen to be affected in tri, occurs normally - this seems also to be the case with the injected embryos, the exceptions being those embryos incubated at 22°C in which the shortened body axis would suggest an additional effect on convergent extension. It is known that the tri mutation displays a cold-sensitive aspect with respect to the degree of cyclopia seen,
so it is likely that the cell movements during convergent extension in the injected embryos could be similarly affected.

4.3.2.5 Interfering with the non-canonical Wnt pathway gives a similar phenotype

*slb* has recently been mapped and found to be a loss-of-function allele of the zebrafish *Wnt11* homologue (Heisenberg et al., 2000). This gene was found to be expressed within the non-axial, lateral mesoderm and was found to be required to mediate medio-lateral intercalation of cells along the anterior-posterior axis. Activation or blocking of the canonical Wnt pathway by injections of *dsh* or a dominant negative *Tcf* generated phenotypes unrelated to those seen in *slb*. However, injection of a truncated *dsh* construct containing only the PDZ and DEP domains thought to transduce the Wnt signals involved in morphogenesis and not those of the canonical Wnt pathway was able to rescue the *slb* phenotype; conversely, a truncated dominant negative form lacking also the PDZ domain induced the phenotype in wild-type embryos.

There are thought to be two classes of Wnt genes: those involved in the classical, Wingless-type pathway such as mouse *Wnt1* and zebrafish *Wnt8* and *Wnt8b* (Kelly et al., 1995); and the second, non-canonical class, including *XWnt5A, Wnt11* and *Wnt4* (Ungar et al., 1995). Injection of *Wnt4* or *XWnt5A* gave a phenotype that was almost identical to that seen with VMRho, consisting of cyclopia, a displaced telencephalon and hairpin folds in the hindbrain; there was also occasional shortening of the trunk and tail. These data suggest that it is the positioning of cells that is affected, rather than their identity. The effects of this non-canonical Wnt pathway suggests that there are similarities between the intracellular pathways regulating convergent extension movements in vertebrates and those generating epithelial planar polarity in *Drosophila*. The Wnt-mediated assignment of polarity may indeed contribute to the morphogenetic movements of gastrulation.

4.3.3 Blocking all Rho function has a severe effect on embryogenesis

4.3.3.1 *C3 exoenzyme acts within minutes of being transcribed*

It was decided to block the function of all Rho isoforms within the embryo by the injection of a tagged, synthetic RNA construct - initial trials with direct injection of the protein, or incubation of embryos in a solution of such were unsatisfactory. The rapidity of the response to the injected RNA was somewhat
4. The Role of the RhoA GTPase During Zebrafish Embryogenesis

unprecedented - conventional wisdom did not predict such a swift processing of the construct, such that its morphological effects could be seen within 20 or so minutes.

A similar effect was seen in the injection of fertilised *Xenopus* eggs with C3 protein (Drechsel et al., 1997). The first and all subsequent cytokinetic events were blocked and although the first cleavage furrow progressed bidirectionally as normal around the embryo, it failed to ingress. New membrane was deposited at the furrow as in the untreated embryos, suggesting that Rho function is not required for cell cycle progression or the specification of the position of the cleavage furrow, nor is the vesicular transport of stored membrane to the furrow affected. However, staining with phalloidin revealed attenuated levels of filamentous actin in the cortex of the embryo at the expense of excessive filaments in the cytoplasm: thus, blocking Rho function interferes with localisation of actin to the cortex rather than the regulation of its polymerisation. Rho might be important for maintaining the localisation of an actin-assembly factor to the cortex, the release of which into the cytoplasm is brought about by C3 treatment.

Perhaps more pertinent to the zebrafish results are the effects seen when *Drosophila* embryos are injected with C3 at cellularisation stages (Crawford et al., 1998). The cytoskeleton is disrupted, the nuclei move away from the cortex and there is an eventual halt in embryogenesis - within a few minutes of injection, there is a halt in the invagination of the cleavage furrows and the nuclei become progressively disorganised in a wave which radiates away from the initial injection site. Looking at the zebrafish time-lapse series (fig. 4.14) a similar process appears to be occurring. The cleavage furrows become less pronounced than in the untreated embryo and by eye, it is no longer possible to pick out individual cells, although they can be seen in fixed tissue and appear larger than normal, with the whole embryo being exceedingly fragile. The spreading of this effect throughout the embryo may well be due to the contribution of the injected RNA to daughter cells, or there may be some degree of active transport of the C3 protein between cells. However, although in the normal scheme of events the cells are no longer connected to each other after the 16 cell stage, the apparent loss of cellularisation or fragility of the cells may allow the toxin to travel freely throughout the embryo. Immunohistology for the presence of phosphorylated histone, a marker of active cell division, reveals that nuclear division is occurring in the absence of Rho function, although there may be some effect on the condensation of the chromatin - Rho may be required for the nuclear architecture and the formation of the mitotic spindle in zebrafish but if so, this is a minor effect compared to the other aspects of the phenotype.
4. The Role of the RhoA GTPase During Zebrafish Embryogenesis

4.3.3.2 *C3 does not prevent gene transcription*

*In situ* hybridisation on C3 RNA injected embryos revealed that there is no disruption to the distribution of transcripts of ubiquitously expressed genes - *zRac1* is found to be expressed throughout the embryo as it is in the wild-type. It must be noted though, that there is no way of distinguishing these transcripts as being maternally contributed or transcribed zygotically and hence there is no way of ascertaining whether this factor would make a difference in the maintenance of transcript distribution. There is also a report that C3 has an effect in downregulating the expression of the *RhoB* transcript in chick - it would be interesting to see if similar effects are seen on the expression of other Rho GTPases in zebrafish, if such probes were available (Liu & Jessell, 1998). Likewise, it would be interesting to see whether the expression of *MyoD* is extinguished as it is in C3 treated mouse myoblasts (Carnac et al, 1998) - unfortunately, no C3 injected zebrafish embryo has developed long enough for this to be a viable assay.

One of the most important stages in zebrafish embryogenesis is symmetry breaking. By the 50% epiboly stage, a trained eye can pick out deviations within the previously spherical embryo and thus the beginnings of the establishment of polarity. Pre-compaction mouse embryos cultured in medium containing C3 fail to develop an apical pole and compaction fails to occur correctly with a loss of adhesion between the blastomeres - this effect is somewhat reversible though, as when C3 is removed from the medium, adhesion is reestablished but cytokinesis remains blocked, giving rise to binucleate blastomeres (Clayton et al, 1999). Likewise, there is a loss of polarity, or at least cell identity, in C3 RNA injected zebrafish embryos, as judged by the expression of the mesodermal marker *goosecoid*. In the wild-type, the expression of this gene marks the cells which are involuting at the shield and so is specific for dorsal, hypoblastic cells. However, in the C3 treated embryos, expression of this gene is superficial and in random arrays of cells on the surface, implying that C3 treated embryos do not generate a dorso-ventral axis, nor do the cells undergo the movements of gastrulation and epiboly.
4.4 Conclusions

The Rho GTPases are required during zebrafish embryogenesis for the generation of polarity and the cell movements of convergence and extension. These processes are probably dependent on an intact, dynamic actin cytoskeleton regulated by Rho, but there is additional evidence to suggest that RhoA mediates signalling through the non-canonical Wnt pathway to bring about convergence and extension of the axial mesoderm.
5. The characterisation of gimpy

CHAPTER FIVE

The characterisation of gimpy, a mutation recovered from an ENU mutagenesis screen

5.1 Introduction

The zebrafish has been used as an in vivo model for vertebrate development for several years. Its rise in popularity as the genetic organism of choice in many labs has been facilitated by the powerful approaches available for identification of novel genes involved during embryogenesis. Gene targeting in the mouse by homologous recombination in ES cells allows the function of most cloned genes to be assayed (Capecchi, 1989) but this is a costly and time-consuming process - a more efficient way to find novel genes is to screen for them directly by examining phenotypes generated by mutagenesis screens. In 1980, a landmark paper was published, describing a systematic search for such genes in Drosophila melanogaster (Nusslein-Volhard & Wieschaus, 1980) closely followed by work suggesting a similar approach could be taken in the zebrafish (Streisinger et al., 1981). Nearly two decades on from this pioneering work, an ENU mutagenesis screen was carried out in the zebrafish research group at University College London and one of the mutants I identified during the course of that screen, gimpy, is described here.

The notochord is the skeletal element necessary for locomotion in lower chordates and the development of the notochord is a critical feature of larval stages in lower vertebrate embryogenesis. A transient structure, it provides mechanical support prior to the development of cartilaginous elements and an ossified vertebral column as well as being a signalling centre for the patterning of the ventral neuroectoderm and paraxial mesoderm. Being composed of a single cell type, the notochord is a simple model for studying organogenesis as it undergoes all of these processes involved therein, such as cell differentiation, proliferation and migration. As it develops, it becomes surrounded by a thick layer of extracellular matrix which contributes to the fibrous collagen sheath providing mechanical support to the turgid notochordal column (Eikenberry et al., 1984; Lauscher & Carlson, 1975).

10% of the 700 mutations maintained from the Boston mutagenesis screen in the 1990s affected the development of the notochord. Amongst these, the mutations can be classified with respect to the stage of development they affect - in some cases, the mutations affect the specification of the chordamesoderm, the notochord antecedent which is surrounded by presumptive muscle tissue, at 12hpf: for instance,
in bozozok (Solnica-Krezel et al., 1996) and floating head (Talbot et al., 1995), the chordamesoderm is absent leading to the complete loss of a notochord, amongst other phenotypic abnormalities. Later in development, the cells in the chordamesoderm differentiate and a large vacuole forms within each, giving rise to the characteristic 'stack of pennies' appearance to the notochord which act as a protoskeletal rod running the length of the embryonic axis. Likewise, a number of mutations affecting this process were uncovered in the Boston screen and found to fall into two further categories. The first group were found to affect notochord differentiation, e.g. bashful, no tail; the second, notochord maintenance, e.g. changeling, maggot (Stemple et al., 1996). Of these two, the first group is seen to have more severe, pleiotropic phenotypic effects and it is about this 'dwarf' category, specifically the mutations sleepy (sly), bashful (bal), and grumpy (gup) that more shall be said.

The mutations give similar phenotypes, with sly and gup being very similar and bal being weaker, and for each of the mutations, a large number of alleles of varying severity is known - at least 12 for gup, 19 for sly and 26 for bal. The failure of notochord cells to vacuolate appears to be the primary defect for each mutation as mentioned above, but accompanying this is a disorganisation in the patterning of the brain, a lens which is extruded from the retinal cup in the eye and, in the trunk of affected animals, a failure in the formation of chevron-shaped somites with U-shaped somites replacing them.

5.2 Results

5.2.1 Identification of the gimpy mutation

During the course of an ENU mutagenesis screen carried out at University College London, the following mutant line was found in a screen of the F3 progeny of ENU-treated founder males. The mutation was identified on screening clutches of embryos for morphological defects at 24hpf and one pair of carriers was identified from the F2 family with a subsequent re-screen of the family identifying another carrier pair. The phenotype was found to be one in which the primary morphological defect is the failure of the notochord to vacuolate and differentiate during the late-somitogenesis stages of development (ca. 20hpf). Accompanying this is a range of secondary defects including aberrant morphology in the brain, protruding eyes and a shortened body axis - it is on account of this final point that the mutant was
categorised as one of the 'dwarf' class of mutants (Stemple et al., 1996) and given the name *gimpy* (*gip*).

### 5.2.2 Embryonic morphology

#### 5.2.2.1 Live examination of embryos reveals a number of defects

The *gimpy* phenotype is clearly discernible from 20-22hpf, even at a gross level (compare wild-type embryos in fig. 5.1a with mutants in fig. 5.1b). The embryos are clearly shorter than their sibs, with a reduced yolk plug extension and somites which are narrowed in their anterior-posterior aspect (compare fig. 5.1c and fig. 5.1d). A more detailed examination reveals the detail of the neural and notochordal defects. The midbrain-hindbrain boundary is shifted anteriorly in *gip* (fig. 5.2b), although this may be a function of the overall shortening of the body, and the vesicles of the hindbrain appear to be swollen and more conspicuous (arrowheads, fig. 5.2b). The developing lens protrudes from the presumptive retina (fig. 5.2d) and the shape of the eye is irregular compared to wild-type sibs. Finally, using Nomarski optics, the notochord phenotype is obvious at 24hpf. The notochord precursor cells fail to vacuolate (seen as the 'pits' in the notochord cells, fig. 5.2e) and no discrete boundary forms between the notochord and the dorsal and ventral tissue flanking it (compare figs. 5.2e and 5.2f). At this stage, *gip* embryos are capable of movement by means of a mild shuddering when touched, but they fail to display the stereotyped writhing of their wild-type sibs.

By 48hpf, the mutant embryos are beginning to degenerate. The circulatory system fails to form correctly, leading to embryos with varying degrees of heart and tissue oedema. The embryos lose their spontaneous movements and become paralysed and the pectoral fin buds fail in their outgrowth, remaining as stumps. However, pigmentation forms as for the wild-type, suggesting that some migration of the neural crest is occurring normally. Death occurs between three and six days after fertilisation, with the oedema gradually worsening and embryos becoming necrotic - those surviving past five days would never be viable as their jaw defects, coupled with their paralysis, would mean that they could not feed.
Fig. 5.1 The *gimpy* mutation becomes discernible by 24hpf

a) Wild-type sibs  
b) *Gimpy* mutants  
c), d) higher power magnification live photos of embryos in a), b)

The *gimpy* mutant has a dwarf phenotype, such that embryos are smaller than their wild-type sibs (compare lengths of yolk plug extension, c, d). The somites, s, are also narrower with a less well-defined shape.

s somites y yolk plug extension

Scale bars - a) - d) 100μm
Fig. 5.2 The primary morphological defects in the *gimpy* mutant at 24hpf

a) wild-type sib, lateral view  
b) *gimpy*, lateral view  
c) wild-type sib, antero-dorsal view  
d) *gimpy*, antero-dorsal view  
e) wild-type sib, lateral trunk  
f) *gimpy*, lateral trunk

Arrowheads in b show swollen hindbrain vesicles in the mutant. The distance between the eye, e, and the midbrain-hindbrain boundary, mhb, is reduced in *gimpy* compared to sibs. Additionally, the lens, l, appears to protrude in the mutant and the notochord, nc, fails to vacuolate.

**e** eye  
**l** lens  
**mhb** midbrain-hindbrain boundary  
**nc** notochord

Scale bars – a)- d) 50μm; e), f) 25μm
5.2.2.2 The ultrastructure of the notochord is severely disrupted

To examine further the notochordal phenotype, transmission electron microscopy was carried out on ultrathin, transverse sections cut from comparable regions of the trunk in both wild-type and gimpy embryos. Even at a lower power (figs. 5.3a, b) the disruption in the mutant is clear. In the wild-type, there is a large, open region in the notochord (fig. 5.3a) traversed by the membranes of the vacuolated cells. Looking at a higher magnification, there is an outer layer of cells with numerous mitochondria and endoplasmic reticulum surrounding the vacuolar cells (data not shown). The whole structure is encompassed by the notochordal sheath, composed of densely packed collagen II fibres (Eikenberry et al., 1984) wrapped in an elastic sheath. However in gimpy, the morphology described for the wild-type has all but disappeared. There is a remnant of a vacuole which is meagre compared to the wild-type (fig. 5.3b) and the clearly defined subcellular and supporting structures are lacking, having been replaced by amorphous, dense debris - even at a high magnification (data not shown), no distinct structure is apparent and only an insignificant attempt at making the notochord sheath is visible (fig. 5.3b).

5.2.3 Patterning of the central nervous system

5.2.3.1 Immunostaining for anti-acetylated tubulin reveals severe defects in the axon scaffold and the embryonic brain

As might be expected from the morphology of the brain, there is considerable mispatterning of the axon scaffold at 48hpf, as shown by the expression of the acetylated tubulin epitope (Wilson et al., 1990). The telencephalon is reduced and shifted ventrally in gimpy and, strikingly, the tectum appears to be absent aside from a few stray projections. The trigeminal ganglion, however, appears to be correctly positioned, taking into account the smaller size of the embryo (figs. 5.4a, b). The major tracts crossing the midline are affected though - their positioning appears to be normal in gimpy, but there is defasciculation, such that the anterior commissure and the tract of the post-optic commissure are less-tightly bundled (figs. 5.4c-f). The lateral line projection is also missing.

The swollen, over-vesiculated hindbrain has a surprising neural phenotype when examined using this technique. It could be proposed that, since the hindbrain appears to be more segmentally organised than usual, this would be reflected in strictly arranged arrays of hindbrain commissural interneurons. However, this is
5. The characterisation of *gimpy*

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**Fig. 5.3** *gimpy* mutants have a disorganised notochord at the ultrastructural level

a) wild-type sib, transverse section  
b) *gimpy* mutant, transverse section

There are radical and startling differences between the *gimpy* mutant and the wild-type sib at 48hpf. The notochord (**n**), a region with large expanses of space in the wild type (a), has failed to inflate in the mutant and is surrounded by structureless, darkly stained debris (b). A striking defect in the mutant is the lack of the notochord sheath (**ns**), a thick fibrous layer comprising the collagenous sheath (**cs**) and the outer elastic sheath (**es**), which circumscribes the entire notochord in the wild-type. The mutant has attempted to make this structure, but it is incomplete and in disarray (white asterisk, b).

**mf** muscle fibres **ns** notochord sheath **nu** nucleus **v** vacuole

Scale bars - a), b) 2μm
5. The characterisation of *gimpy*

Fig. 5.4  **Immunostaining using anti-acetylated tubulin shows defects in the developing CNS at 48hpf**

a) wild-type sib, lateral view  
b) *gimpy*, lateral view  
c) wild-type sib, dorsal view  
d) *gimpy*, dorsal view  
e) wild-type sib, ventral view  
f) *gimpy*, ventral view  
g) wild-type sib, lateral trunk  
h) *gimpy*, lateral trunk

*gimpy* mutants show a general disorganisation of the CNS at 48hpf. Many tracts appear to be defasciculated - the anterior commissure (wild-type, e; *gimpy*, f) and the spinal cord (g compared to h) being prime examples. Additionally, some tracts are reduced in size in the mutants - the optic nerve is affected particularly severely.

**AC** anterior commissure, **LLP** lateral line projection, **MN** motor neurons, **ON** optic nerve, **RB** Rohon Beard cells, **SC** spinal cord, **TecN** tectal neuropile, **Tel** telencephalon, **Tri** trigeminal ganglion, **TPOC** tract of the post-optic commissure, *hindbrain commissural interneurons.

Scale bar - 50µm throughout
5. The characterisation of \textit{gimpy}

clearly not the case (fig. 5.4d) - although there remains some level of order, in the
main the hindbrain interneurons are chaotic.

5.2.3.2 \textit{The Mauthner neurons in the hindbrain fail to project correctly}

The 3A10 antibody can be used to stain specifically the developing
projections of the Mauthner neurons, one of the pairs of reticulospinal neurons in
rhombomere 4 of the hindbrain, whose axons project towards the ventral midline and
then decussate to pioneer the contralateral medial longitudinal fasciculus
(Mendelson, 1986). Fig. 5.5 shows that in \textit{gip}, this process does not occur as it
should - the cell bodies of the Mauthner are oddly shaped and they fail to project as
they ought. Axons cross the midline but deviate somewhat after that, with ectopic
projections also being present.

5.2.3.3 \textit{In situ analysis reveals neural gene expression to be largely wild-type}

To try to ascertain the genetic basis underlying the neural aspect of the
phenotype, a series of \textit{in situ} hybridisations was carried out on \textit{gip} embryos and their
wild-type sibs at from late somitogenesis stages (when mutants can be first identified
and separated out) to 32hpf. However, in the main, a surprising result is seen - for
the markers tested, for instance \textit{Ephrin-B2a} (Durbin et al., 1998), \textit{DeltaD} (Haddon et
al., 1998), \textit{fgf8} (Reifers et al., 1998) the expression patterns are the same in the
mutant as for the wild-type (data not shown). However, to qualify these results, it
must be noted that in some cases, the domains of expression may have a slightly
altered shape, but this is thought to be more a reflection of the underlying anatomical
differences in the \textit{gip} embryos rather than any true change in gene expression
boundaries and hence altered genetic regulation - see fig. 5.8 for examples of this
with respect to this regarding the transcription factor \textit{pax2} (Krauss et al., 1991).

There is one exception found to this - the expression of \textit{krox20}, a marker for
rhombomeres 3 and 5 (r3, r5) in the hindbrain (Oxtoby & Jowett, 1993). At early
stages in development, the gene is expressed in two parallel stripes, but as the
hindbrain matures, the expression domain separates into four squares, in r3 and r5 to
either side of the midline (fig. 5.6a). In \textit{gip}, this separation fails to go to completion,
leaving residual bridges of \textit{krox20} expressing cells spanning the midline (fig. 5.6b).
These bridges are not stereotyped - that is to say, the extent to which the bridging
occurs varies between embryos and between stripes in the same embryo.
5. The characterisation of *gimpy*

**Fig. 5.5 Immunostaining using the 3A10 antibody at 26hpf**

a) wild-type embryo, dorsal flatmount  
b) *gimpy* embryo, dorsal flatmount

Arrowheads demarcate the decussation of the Mauthner neurons prior to contralateral projection. Asterisk in b) marks an ectopic axonal projection in the mutant embryo.

Scale bars - a), b) 25μm
Fig. 5.6 *Krox20* expression at 26hpf reveals hindbrain morphology defects in *gimpy*

a) wild-type, dorsal flatmount  
b) *gimpy*, dorsal flatmount

*krox20* is expressed in rhombomeres 3 and 5 in the developing hindbrain. By 26hpf, its domains have become separated across the midline to appear as four, separate zones. However in *gimpy*, the separation is incomplete and bridges of tissue (indicated by arrowheads) remain between the two halves of the rhombomere in both r3 and r5.

Scale bars - a), b) 50μm
5. The characterisation of *gimpy*

5.2.4 Patterning of the eye

5.2.4.1 Immunostaining reveals defects in the projection of the optic nerve and the layering of the retina

The eye morphology phenotype in the mutant translates at the axonal level to a severe reduction in the thickness of the optic nerve, with fewer projections feeding into it from the retina cupping the lens (compare fig. 5.7f with fig. 5.7e). The optic chiasm is apparently absent, with neurons failing to project contralaterally to innervate the tectum.

To look more specifically at the projections within the eye and the retina, the antibody zn-5 is particularly useful for illuminating the layering and exit of the axons of the eye. In the wild-type, the retinal ganglion cell layer is cleanly defined and neurons pass through it at one point only (the choroid fissure) to form the optic nerve (fig. 5.7c). The optic nerves from both eyes meet at the midline to form the optic chiasm, the crossing point at which the majority of the neurons will project to the contralateral side of the tectum and a few to the ipsilateral side (fig. 5.7e). In *gimpy*, this is clearly not the case (figs. 5.7d, f). The retinal ganglion cell layer is mossy and has spread to cover a large proportion of the eye territory, with the optic nerve being fed by projections from across the retina. Looking at the optic chiasm, there is an almost complete failure to project contralaterally, with a few stray axons apparently travelling rostrally in an aberrant fashion.

5.2.4.2 Expression of *pax2.1* is not abnormal

To see whether the optic nerve phenotype seen in *gimpy* has some basis at the level of gene expression, *in situ* were performed to look at the patterning within the eye field at 24hpf, i.e. a time after the specification of the eye and the fields within it, but before the differentiation of any neural tissue or axonal projections. *pax2.1*, a marker of presumptive optic stalk tissue, is not expressed abnormally in *gimpy* embryos - it is shifted slightly towards the midline, but it is not lacking (if anything the domains of expression may be a tad expanded) and so is not the cause of the reduced optic nerve phenotype (fig. 5.8d). Likewise, there is no disruption in the expression of *pax6* (Macdonald et al., 1994) (data not shown) suggesting that the neural retina tissue is specified correctly. Neither of these genes' expression patterns account for the later disorganisation seen in the visual system, implying that the phenotype is caused by a factor required after the initial specification of optic stalk vs. retinal tissue has occurred.
5. The characterisation of *gimpy*

**Fig. 5.7** The zn-5 antibody shows severe defects in the eye and aberrant staining in the trunk at 48hpf

a), c), e) wild-type sib, ventral view
b), d), f) *gimpy*, ventral view

Immunostaining with the zn-5 monoclonal antibody reveals a number of defects in the *gimpy* mutant when compared to wild-type sibs. The retinal ganglion cell layer appears to be disrupted, with the projections being 'mossier' than in the wild-type. The optic chiasm is also affected, with the majority of axons failing to make their contralateral projections correctly. The boxed areas in c, d are areas magnified in e, f.

*oc* optic chiasm, *rg* retinal ganglion cells

Scale bars - a), b) 50μm; c)-f) 25μm
5. The characterisation of *gimpy*

**Fig. 5.8** Expression of *pax2* in *gimpy* embryos at 24hpf

a) wild-type sib, anterior lateral view  
b)*gimpy*, lateral anterior lateral view  
c) wild-type sib, dorsal flatmount  
d) *gimpy*, dorsal flatmount  
e) wild-type sib, posterior lateral view  
f) *gimpy*, posterior lateral view

At a gross level, the expression of *pax2* in *gimpy* embryos is not unusual. However, there are subtle differences in the morphology of the expression domains. From a lateral aspect, the expression domains in the optic stalks are expanded antero-ventrally in the floor of the diencephalon (see white asterisks in a). Viewed dorsally, these domains appear more compact and shifted medially compared with the wild-type sibs (black arrowheads, c and d). The midbrain-hindbrain boundary (*mhb*) domain appears more compact in the mutant. Expression in the nephric ducts (*nd*) appears normal. However, the spinal interneurons expressing *pax2* (black asterisks, e and f) are closer together with ill-defined margins of expression.

*mhb* midbrain-hindbrain boundary, *nd* nephric ducts

Scale bars - 50µm throughout
5.2.5 The expression of sonic hedgehog suggests a signalling capacity in the undifferentiated notochord tissue

ssh is known to be an important source of signalling information in the developing embryo (Krauss et al., 1993) and its sites of expression reflect this, particularly in the developing notochord and floorplate. The expression of ssh as ascertained by in situ hybridisation at 24hpf reveals more surprising information about the gip mutant embryos. Expression is virtually indistinguishable from that in wild-type sibs in anterior structures such as the hypothalamus and pituitary gland, and the dorsal and ventral regions of the diencephalon, whereas there appears to be a slight reduction in expression in the ventral mesencephalon (see fig. 5.9e and f).

It is in the notochord and floorplate domains of expression that the gip mutants are remarkable, in that ssh levels are perfectly normal in both structures and in the former, the progressive rostro-caudal downregulation of expression is seen in the mutant as it is in the wild-type (figs. 5.9g-j). This would suggest that in spite of being undifferentiated and functionless as a mechanical support to the embryo, the notochord retains at least one of its signalling capacities and should act upon the ventral neural tube and surrounding sclerotome accordingly (Fan & Tessier-Lavigne, 1994; Macdonald et al., 1995). This result also implies that the lack of notochord differentiation is not due to the mis-specification of the chordamesoderm, nor is it due to the death of the notochord precursors as they are clearly alive at 24hpf (fig. 5.9j).

5.2.6 By 48hpf gimpy mutants become paralysed

One of the striking aspects of the gip phenotype is the gradual paralysis of the embryo. Even at the age of 20-24hpf, when the wild-type sibs are making their first spontaneous movements within the chorion, the mutants seem to lag behind and only manage a vague 'shuddering' rather than the twisting and turning usually seen at this stage. As mentioned above, even this movement is gradually lost and the embryos require assistance in hatching without ever gaining the ability to swim freely. This aspect of the phenotype could result from a defect at any point in the stimulus-reception—signal transduction—motor response pathway so some of the more tractable points were investigated.
5. The characterisation of *gimpy*

**Fig. 5.9** *sonic hedgehog* expression is normal in *gimpy* mutant embryos at 26-28hpf

a) wild-type sib, 28 hpf, lateral view  
b) *gimpy*, 28hpf  
c) wild-type sib, 26hpf, dorsal flatmount  
d) *gimpy*, 26hpf  
e) wild-type sib, 26hpf, anterior lateral view  
f) *gimpy*, 26hpf  
g) wild-type sib, 26hpf, posterior lateral view  
h) *gimpy* 26hpf  
i), j) enlargements of boxed regions in g), h) respectively

There are subtle differences in the expression pattern of *shh* in the mutant compared to the wild-type. Expression in anterior structures such as the hypothalamus and pituitary gland, and the dorsal and ventral regions of the diencephalon is normal, however there appears to be a slight reduction in expression in the ventral mesencephalon (see e and f). Expression in the notochord and floorplate is also normal.

**di** diencephalon, **fp** floorplate, **hy** hypothalamus, **me** mesencephalon, **nc** notochord

Scale bars – a), b) 100µm, c)-h) 50µm, i), j) 25µm
5.2.6.1 Motor neurons project aberrantly within the somites

Hypothetically, the paralysis seen at 48hpf may result from a failure of the motor axons to stimulate the somitic musculature. Again using the anti-acetylated tubulin antibody, it is seen that this may well be a contributory factor to the immotility as the primary motoneurons appear to be projecting straight ventrally from the spinal cord between the somites rather than projecting midway between the anterior and posterior somite boundaries with a caudal deviation when the horizontal myoseptum is reached (figs. 5.4g, h). In addition, the gip spinal cord is defasciculated compared to the wild-type, but it is difficult to speculate as to whether either of these factors could be responsible for the total paralysis seen.

5.2.6.2 The distribution of muscle myosin is defective in gimpy muscles

Alternatively, the paralysis phenotype may be being caused by an inadequacy in muscle formation. Expression of the muscle precursor marker MyoD (Weinberg et al., 1996) is normal in gip embryos up to late-somitogenesis (data not shown), suggesting that the initial specification of muscle cells is normal. However, immunostaining using the pan-myosin antibody (gift; Dr. T. Schilling, UC, Irvine) reveals a different story at 26hpf (fig. 5.10). In the wild-type larvae, the chevron-shaped somites have a clearly visible horizontal myoseptum (fig. 5.10a) and a regular array of striated muscle fibres, with an even distribution of myosin (fig. 5.10e). In the mutant however, there is a clear reduction in staining in the dorsal and ventral edges of the somites which becomes more pronounced moving posteriorly down the tail (fig. 5.10b). The somites only sporadically possess a horizontal myoseptum, and they appear to be more u-shaped than chevron-shaped (though not to the same extent as the mutants categorised by the presence of u-shaped somites, such as you or uboot (van Eeden et al., 1996).) At a higher magnification, it appears that both the myofibrils and the distribution of myosin are disrupted: the fibrils seem to occupy far less space within the somite. There appears to be the same number of fibres present in the wild-type muscles, but each fibre is much thinner than it ought to be and the fibres appear not to be as tightly bundled (fig. 5.10f). The myosin present is distributed in an aberrant way, such that 'clumps' of it are visible at the somite boundaries but less can be seen along the length of each fibre.

By 48hpf, this state of affairs appears to be partially resolved, in that the amount of myosin present looks to have been upregulated, with a more even distribution along the fibres (fig. 5.10c, d; g, h). The fibres themselves though are still in a state of disarray - they are much narrower than their wild-type counterparts.
5. The characterisation of *gimpy*

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**Fig. 5.10** Immunostaining using the pan-myosin antibody reveals severe muscular defects

a), e) wild-type sibs, 26hpf  
b), f) *gimpy* 26hpf  
c), g) wild-type sibs, 46hpf  
d), h) *gimpy* 46hpf  

At 26hpf, the muscle fibres in *gimpy* appear to be less tightly packed and have an aberrant myosin distribution compared to their sibs. There is a clear loss of striation in the myofibrils. By 46hpf, the amount of myosin present seems to have recovered to wild-type levels, however the fibres appear loose and the horizontal myoseptum (asterisks, g) is absent.

Lateral views: anterior is to the left, a) - d); anterior is to the top, e) - h)

Scale bars - a) - d) 50μm; e) - h) 25μm
and lack striations. The fibres within a somite do not seem to be correctly adhered to each other, giving the appearance of the muscle fibres literally falling apart.

5.2.6.3 The ultrastructure of the somite reveals mistargeting of myofibrils

To investigate more thoroughly the muscle phenotype, transmission electron microscopy was carried out as before on comparable trunk sections of wild-type and gip mutant embryos at 48hpf (fig. 5.11). In the sections observed, the absolute numbers of myofibrils present does not appear to differ between the wild-type and the mutant, but their arrangement does. In the wild-type, only transverse sections of fibres were found, corresponding to the plane of sections cut; conversely in gip, fibres were found within the same section that traversed the somite both obliquely and in a medio-lateral as opposed to an anterior-posterior direction (compare figs. 5.11a and c). On close inspection, these longitudinal fibres were deemed to be wild-type in terms of their acto-myosin arrangement, displaying text-book arrays of Z- and M-discs. Confirming this was the regular array seen in the transverse sections - the crystalline arrangement of myosin cables surrounded by actin filaments was found in gip as it is in the wild-type (figs. 5.10b and d). However, a conspicuous difference between the two cases is the reduction in the amount of connective tissue holding the myofibrils together - this may well account for the ragged appearance of the fibres seen with the pan-myosin antibody.

Taken as a whole, neither the nervous system nor the musculature can be deemed to be the sole cause of the paralysis displayed by the mutant, but given the deficiencies seen in both, that the paralysis occurs is barely surprising.

5.2.7 gimpy embryos have an aberrant body growth phenotype

gimpy mutant embryos are smaller than their wild-type sibs, but what is the basis of this dwarfism? There could be several reasons - an excess of cell death in the embryo, a reduction in the signals required for cell growth and, leading on from this point, a failure of the basic bodily elements to grow to their full extent. To address this feature of the phenotype, several experiments were carried out.

5.2.7.1 The pituitary gland develops normally in gimpy embryos

A possible cause of reduced growth would be the failure of the pituitary gland to differentiate and secrete the hormones necessary for development (Glasgow et al., 1997). In situ hybridisation using the lim3 marker for pituitary
5. The characterisation of *gimpy*

**Fig. 5.11** Anterior-posterior muscle patterning is disrupted in *gimpy* at 48hpf

a), b) wild-type sib, transverse section  
c), d) *gimpy* mutant, transverse section

In *gimpy*, the somitic musculature is disrupted compared to the wild-type. Transmission electron micrographs of transverse sections shown here demonstrate that in the wild-type, only fibres cut in this plane are seen (tf) whereas in the mutant, myofibrils are seen to project aberrantly, shown by the presence of obliquely- and longitudinally-sectioned fibres (of and lf).

Also in the mutant, there is a reduction in the density of connecting fibres (c) holding the myofibrils within each bundle.

- c connecting fibres  
- lf longitudinal fibres  
- of oblique fibres  
- mt mitochondrion  
- tf transverse fibre

Scale bars - a), c) 1μm; b), d) 100nm
differentiation was carried out on 24hpf embryos (data not shown) and the expression pattern is found to be indistinguishable from that in the wild-type. This suggests that the dwarfism and later deterioration of the gip embryos does not have a hormonal basis.

5.2.7.2 The reduced body length does not result from an increase in cell death

It could be speculated that an increased level of cell death in the embryo could lead to a smaller body developing, if that increase occurs uniformly throughout the embryo, such that all parts of the body are affected and not merely one organ or region. To look at the levels of cell death in gimpy, Acridine Orange staining was performed on 24hpf embryos and their wild-type sibs (fig. 5.12). When viewed under fluorescent lighting conditions to illuminate the dying cells, it appears that the mutant embryos have very similar levels of cell death as their sibs (compare panels 5.12c and d) with no obvious increase above background levels. Additionally, there is no evidence of cell death in the undifferentiated notochord, supported by the shh result described above.

5.2.7.3 gimpy embryos have severe craniofacial defects

One of the earliest skeletal features to develop is the craniofacial system, composing the branchial arches and the mandibular elements of the developing jaw. By 5dpf, the individual cartilaginous elements have differentiated, extended, and can be stained using the dye Alcian blue. gimpy mutants have obvious disruptions in this system (fig. 5.13) - although each individual element is present, such as arches P3-P7, they do not appear to be anchored in the midline and project medio-laterally rather than extending obliquely in an anterior-posterior manner as in the wild-type (figs. 5.12e, f). Meckel's cartilage, the palatoquadrates and the basihyal, the cartilages of the lower jaw, fail to project anteriorly (compare fig. 5.13b, c) and hang ventrally, giving the embryo the appearance of a smaller head with a 'gaping' mouth. This latter feature increase the distance between the lower jaw and the more dorsal neurocranium, so much so that the two appear in different focal planes rather than being 'merged' as in the wild-type. (fig. 5.13h, i).

From the data seen here, it would seem that the gip phenotype is not due to a disrupted hormonal level, nor is it caused by an excess of cell death. There is also a phenotypic effect on the structure of the developing skeletal elements.
Fig. 5.12 Acridine Orange staining reveals normal levels of cell death in *gimpy* at 24hpf

a), b) wild-type sib and *gimpy* embryos, lateral view, DIC optics

c), d) wild-type sib and *gimpy* embryos, lateral view, FITC fluorescence

Staining with the Acridine Orange reagent reveals similar levels of cell death (seen in c) and d) as white flecks) in *gimpy* mutants, suggesting that this is not the underlying cause of the dwarfism. There is no extraneous cell death in the undifferentiated notochord cells.

Scale bar - 50µm throughout
5. The characterisation of gimpy

Fig. 5.13 Craniofacial defects in gimpy shown by Alcian Blue staining at 5dpf

a), d), g), Camera lucida drawings of pharyngeal skeleton in lateral (a) and ventral (d) views and neurocranium (g) (adapted from Schilling et al, 1996)
b) wild-type embryo, lateral view
c) gimpy embryo, lateral view
e) wild-type embryos, ventral view
f) gimpy embryos, ventral view
h) wild-type embryo, neurocranium
i) gimpy embryo, neurocranium

In the gimpy mutant, there is a reduction in size of all the cranio-facial elements. Meckel’s cartilage fails to project as far anteriorly as in the wild-type (compare e with f) and the palatoquadrate and basihyal elements are aberrantly positioned (see b and c). In addition, the branchial arches P3-P7 are shaped incorrectly and lack the attachments present in the wild-type. The neurocranium is also affected in the mutants, albeit to a lesser extent in terms of morphology - however, there appears to be an increased dorso-ventral distance between this and the other cartilaginous structures, as visualised by the clear distinction in focal planes between the two in the mutant.

Key: me Meckel’s cartilage (dark green); pa palatoquadrate (dark blue); ce ceratohyal and ba basihyal (yellow); in interhyal (light blue); hy hyosimplectic (red); P3 including ceratobranchial, hypobranchial and basibranchial (pink); P4 (orange); P5 (light green); P6 (purple); P7 (black); ne neurocranium (grey).

Scale bars - b), c), e), f), h), i) 100μm
5.2.8 *The ultrastructure of the skin is greatly disrupted*

At an anecdotal level, it was noticed that the *gimpy* embryos were more sensitive to enzymatic treatments during immunostaining and *in situ* hybridisation than their wild-type sibs, such that they would require shorter trypsinisation and Proteinase K treatments. Transmission electron micrographs of the periderm from the trunk of 48hpf embryos revealed some startling insights to this phenomenon (fig. 5.14). *gip* have a greatly thickened keratinized surface layer, with grossly expanded microspikes (compare figs. 5.14a, b). They are also lacking a basal lamina under this outer epidermal layer. Although this result does not explain the heightened susceptibility to enzymatic treatment, it is an interesting aspect of the phenotype; indeed, the lack of a basal lamina, coupled with the missing notochord sheath, provides clues as to the nature of the underlying genetic defect.
5. The characterisation of *gimpy*

**Fig. 5.14** Transmission electron micrographs of wild-type and *gimpy* mutant periderm at 48hpf

a) wild-type sib, transverse section, trunk
b) *gimpy* embryo, transverse section, trunk

The upper layer of the epidermis is thickened in the mutant embryo compared to the wild-type sib. The microspines (ms) are highly irregular in their morphology

*ms* microspines

Scale bars - a), b) 1μm
5.3 Discussion

5.3.1 Morphological defects of the gimpy mutant

The most striking feature of gimpy is its obvious morphological defects - the embryos are short and peculiar-looking. Closer inspection reveals that they lack a notochord and also have neurological malformations. 24 hours later, the mutant embryos begin to degenerate, losing the ability to move and failing to develop a functioning circulatory system. This rapid decline and ultimate lethality must surely point to a gene that is essential for embryonic development, but also raises issues regarding the onset of the phenotype - until around 20hpf, the mutants are indistinguishable from their sibs, implying that there is no early requirement for the gene in question.

5.3.2 The scaffold of the central nervous system is severely disrupted in gimpy

All of the immunohistological stains performed on these mutants gave results showing severe disruption to the central nervous system in these embryos. Anti-acetylated tubulin revealed a large proportion of the tectum to be missing, and a disorganised array of neurons in the hindbrain, amongst other faults. The latter was somewhat surprising, given the 'over-segmented' morphology of the hindbrain, this went against the expectation that the neurons within would also be in strict segmental array. The arrangement of axons within and leaving the retina was also in disarray, as was the defasciculation seen in the spinal cord.

The phenotype seen using the anti-tubulin antibody at 48hpf does not result from the progressive degeneration in the embryo which begins around that time but is more the elaboration of earlier patterning defects. Examination with this antibody at 28hpf (data not shown) revealed an axon scaffold which is as disordered, if anything more so - the axon tracts in the midbrain are 'mossy' and irregular in appearance and there is a complete loss of any segmentation with respect to axon bundling in the hindbrain. Additionally, the aberrant and non-stereotyped projections of the Mauthner neurons at 26hpf suggests that the mutated gene may be one that is involved in axon guidance, since these and other axons fail to reach their targets correctly. In bashful and sleepy, other dwarf-type mutants, axons leaving the retina fail to project to the optic tectum correctly, either failing to cross the midline and projecting ipsilaterally, or proceeding contralaterally as in the wild-type but then projecting anteriorly rather than posteriorly and into the tectum (Karlstrom et al., 1996). In gimpy, the zn-5 antibody was used to highlight the retinotectal projection.
and it appeared that similar to these other mutants, there was a failure to project contralaterally. *In vivo* injections of DiI into the retina and examination under confocal microscopy would provide a more detailed analysis of this facet of the phenotype.

5.3.3 *The prepattern of the central nervous system appears to be normal*

The defects seen in the axon scaffold could result from a deficit in a pathway required for the establishment of the prepattern and regionalisation of the brain and eye fields. However, *in situ* hybridisation for genes expressed in the nascent CNS such as *shh* (Krauss et al., 1993) and those marking presumptive eye fates, such as *pax2.1* (Krauss et al., 1991) and *pax6.1* (Macdonald et al., 1995) showed gene expression patterns that deviated little from those of the wild-type, with those deviations seen being attributed to incorrect positioning of the expressing cells rather than a change in identity of a given region. This would suggest that the eye and brain phenotypes do not result from an early, patterning or even pre-patterning defect, but arise at some later stage. Results from the sleepy, bashful and grumpy mutants support this assertion, such that the expression of genes affecting the specification of regionality and anterior-posterior patterning, e.g. *rtkl* (Xu et al., 1994) is not affected (D. Stemple, unpublished).

5.3.4 *There is a mild midline defect in gimpy*

There seems to be a mild, variable phenotype involving the midline of the CNS in *gimpy*. As mentioned above, the failure of the retinotectal projection and the mis-routing of the Mauthner point to this, as does the curious *krox20* phenotype, whereby small bridges of tissue are maintained across the midline, joining the two lateral patches of expression in each of rhombomeres 3 and 5. It could be speculated that the gene responsible for *gimpy* is one involved in cell signalling and/or adhesion, rather than being a transcription factor, for example.

5.3.5 *The paralysis phenotype does not have a clear cause*

The mutant embryos show a progressive paralysis phenotype - however, it cannot be stated categorically what the underlying cause for this is. Even before the onset of paralysis, the mutant embryos failed to display the stereotyped, C-shaped escape response to a tactile stimulus (Kimmel et al., 1991), which is mediated by interplay between the Mauthner neuron and the primary motoneurons. In light of the immunohistological data, it is unsurprising that the mutant embryos display a
reduced, shuddering response. The anti-tubulin antibody showed aberrant projections from the primary motoneurons to the somitic musculature, running between rather than into the somites and, would there were a lack of other evidence, this might suggest incorrect firing to the somites and a gradual breakdown in the response from the muscle as the neuromuscular junction fails to be consolidated. There is data to suggest that the muscle fibres themselves fail to form correctly - the distribution of myosin, as elucidated by immunohistology, is uneven along the length of fibres that are loosely packed and in disarray. Coupled with the failure of the myofibrils to traverse the somite properly, as visualised with transmission electron microscopy, it would be hard to imagine such a weakly structured muscle functioning correctly, even with the full neurological input. The paralysis phenotype could well transpire from a combinatorial effect of these two factors. During the course of the U. C. L. screen, another paralysed mutant, akineto, has been isolated and it is in the process of being characterised. However, it is unlikely to be related to gimpy, since the embryos display a complete lack of movement yet are wild-type in all other respects.

5.3.6 Ultrastructural examination reveals a complete collapse of the notochord

As previously mentioned, one of the most distinct features of the gimpy phenotype is the failure of the notochord to differentiate and it is at this stage of development, around 20hpf, that the mutants become distinguishable from their sibs. Unlike the floating head mutant (Halpern et al., 1995), in which there is a shift from axial to paraxial mesoderm and muscle tissue develops in the place of the notochord, the gimpy notochordal cells are specified correctly and express markers such as shh as they do in the wild-type - it appears to be at the time of vacuolation that the deficit becomes apparent. Even after their failure to inflate, the cells still retain their signalling capacity as the neural tube and musculature are effectively wild-type in their pattern. This having been said, the notochord itself is known to be involved in altering the permissiveness of the myotome for motoneuron pathfinding (Beattie & Eisen, 1997) and is involved in growth cone guidance in the spinal cord (Greenspoon et al., 1995). Hence, some aspects of notochordal signalling may be disturbed in gip, the read-out of which is seen as defects in motoneuron guidance in the somites.

It is at the ultrastructural level that a possible underlying cause for the notochord phenotype becomes visible. The open space, peppered with membranes crossing its expanse in the wild-type, is completely lacking in the mutant, being replaced by a much smaller hole surrounded by dense debris. More tellingly though,
the thick, fibrous sheath circumventing the wild-type structure is absent in *gimpy*, as is the basal lamina which lies beneath it, both of which are elements found in embryos of the chick, monkey and human (Camon et al., 1990; Jerome & Hendrickx, 1988; Shinohara & Tanaka, 1988). In *Xenopus*, the internal pressure of the notochord cells rises 2-3 fold as the vacuoles within them increase their osmotic activity and swell. It is at this time that the fibre density in the notochord sheath also increases. It is the resistance of the sheath towards the osmotic swell which permits the internal pressure to rise and hence results in the stiffened rod which can elongate and eventually straighten without being buckled by the surrounding tissues (Adams et al., 1990). Consequently, assuming the process is similar if not the same in the zebrafish, it is easy to see why an embryo lacking the notochord sheath would be unsuccessful in vacuolating its notochord, as is seen in *gimpy*. It would also seem that the unvacuolated cells do not degenerate for at least another 24 hours, if at all - the cells maintain their *shh* expression for as long as their wild-type sibs; and the cell death marker Acridine Orange reveals wild-type levels of dying cells, with no increase in the region of the notochord at 24hpf. Additionally, inspection by eye does not show any regions of necrosis specific to this structure at 48hpf - after this time, the embryos are generally degenerating and so tissue analysis is not possible.

### 5.3.7 The lack of a basal lamina gives clues as to the nature of the mutation

Electron microscopy also revealed the basal lamina underlying the outer layer of the periderm to be lacking in the mutants. This, coupled with the apparent separation of the cartilaginous elements of the jaw and the protrusion of the lens from the optic cup would imply that the mutation lies within a gene encoding a component of the basal lamina, or a gene which is critical for basal lamina assembly or the adherence of cells to it.

### 5.3.8 Identification of the gimpy locus

As mentioned previously, there are several other groups of notochord mutants but perhaps the most relevant here are the three mutants isolated in both the Boston and Tübingen screens, with many alleles found for each - *bashful* (*bal*), *sleepy* (*sly*) and *grumpy* (*gup*), of which *bal* has the weakest phenotype. All three have a phenotype that is similar to each other's and that of *gimpy*, so it would seem likely that *gimpy* is in actuality an allele of one of these genes. Recent complementation testing (D. Stemple, NIMR, Mill Hill) has identified *gimpy* to be an allele of the *sleepy* mutant.
5. The characterisation of *gimpy*

An alternative method of identifying the *gimpy* locus would be to map the mutant. This would involve the positioning of the mutant and any candidate genes onto a genetic map, and determining whether there is any linkage between the mutant and a particular gene (see Postlethwait and Talbot 1997 for a review of this technique). This method has been successfully used to identify genes affected in other zebrafish mutants, for example the *acerebellar* mutant was determined to be a lesion in the *Fgf8* gene (Reifers et al., 1998). The approach used is based on that developed in Pascal Haffter's group in Tübingen.

Firstly, a number of map crosses are established. These are F₁ families generated from the matings of wik strain wild-type fish with known carriers of the mutation, and fin clips are taken from these P₀ founder fish for later PCR-based comparison. Carriers of the mutation from the F₁ generation fish are identified and then used to establish the F₂ generation, of which 30 embryos are used for mapping purposes. Primers used for the identification of simple sequence length polymorphisms (SSLPs) within the zebrafish genome, the genetic location of which are known, are available (Research Genetics) (Knapik et al., 1996) and PCR amplification with these primers can be used to determine which of these markers show polymorphisms in the P₀ map cross fish, and also the pattern of segregation of these polymorphisms in the 30 F₂ generation embryos. When mapping genes, restriction fragment length polymorphisms (RFLPs) are identified for the gene in the P₀ fish of a particular map cross. The segregation pattern of this polymorphism in the F₂ generation is then determined. MAPMAKER computer software (Lander et al., 1987) is used to determine linkage between the RFLP of the gene of interest and any SSLP markers within the given map cross. If linkage is established, the gene of interest can be placed on the zebrafish genetic map and it is then possible to see if the phenotype can be correlated with any candidate genes.

5.3.9 Cloning of sleepy, bashful and grumpy

Work carried out in Derek Stemple's lab at NIMR, Mill Hill has further identified the nature and map position of these mutants. *sleepy* maps to linkage group 2 on the zebrafish genetic map and was found to be a mutation in exon 3 of the *LamC* gene, the γ₁ chain of the Laminin1 protein. Similarly, *grumpy* was found to be a mutation in *LamB1*, the Laminin β₁ chain and *bashful*, *LamA1*, the Laminin α₁ chain, both of which are the other two components of the Laminin1 isoform and which map to LG25 and LG24 respectively.
Laminin is an extracellular matrix protein and a major constituent of all basal laminae. In the zebrafish embryo, the major isoform found is $\alpha_1\beta_1\gamma_1$, also known as Laminin1 and an antibody to this protein shows it to be expressed in the notochord sheath, in the somite boundaries and in the developing brain and retina (D. Stemple, unpublished). sleepy mutant embryos show no immunoreactivity towards this antibody, nor do grumpy, despite each only lacking one of the component chains. However, there is some residual activity in bashful embryos, suggesting that there is a little redundancy of function and possibly explaining the less severe phenotype seen in these mutants.

Another anomaly arises when the autonomy of these mutations is analysed: shield transplants from a donor, epiboly stage embryo will generate a second axis when placed into the ventral side of a host embryo, in what is effectively a chordamesoderm graft. When a wild-type shield is placed into a sleepy host, the second notochord generated is wild-type; when the converse experiment is performed, the additional notochord is phenotypically mutant; thus, the mutation is cell-autonomous. However, when the same experiments are carried out in grumpy, the mutation is seen to be non-autonomous in that a wild-type axis can be generated by the mutant donor cells. This is somewhat puzzling, given that the mutations are apparently of the same nature and raises issues regarding the secretion and assembly of the laminin proteins in the extracellular space - LamC, i.e. sleepy, is thought to be necessary for laminin assembly and laminin is in turn essential for the organisation of other extracellular matrix and basement membrane components in vivo (Smyth et al., 1999).

The lack of laminin1 seems to have less of a detrimental effect on development for the zebrafish than for other species - the embryos become recognisable larvae despite their many defects and are capable of surviving for several days after the mutation has come into effect. This is a stark contrast to the case in the mouse, for example, where a targeted deletion of the LAMC gene results in embryonic lethality at E5.5 and a failure to form basement membrane or differentiate endoderm (Smyth et al., 1998; Smyth et al., 1999). Laminin1 has also been implicated in pathways leading to neurite outgrowth and cytoskeletal clustering in myotubes, both of which are concordant with the phenotypes seen in the laminin-chain mutants and gimpy (Patton et al., 1997). Additionally, laminin reduction or dysfunction has been implicated in certain forms of congenital muscular dystrophy, so these zebrafish mutants may well become a useful therapeutic model for a distressing human disease (Colognato & Yurchenko, 2000).
5.4 Conclusions

The *gimpy* mutant can now be positively identified as a further allele of the *sleepy* mutant, ergo it is a mutation in the *LamC* gene required for the synthesis of the $\gamma_1$ chain of the Laminin1 protein. Consideration of the work carried out on *gimpy* and comparison of its characteristics with those of the other dwarf-like mutants pointed very strongly to the mutated gene being a cell-adhesion molecule, if not another allele of one of the existing laminin mutations and the genetic evidence provided by the complementation testing proved this to be the case.
CHAPTER SIX

General Discussion and Future Directions

In this thesis, two avenues of research have been pursued. Firstly, there was the cloning and expression of two, hitherto unreported members of the Rho family of GTPases in the zebrafish, \( z\text{RhoA} \) and \( z\text{Racl} \). Following on from this, there was the series of experiments addressing the functional role of one of these genes, \( \text{RhoA} \), on embryonic development. Finally, there was the characterisation of \( \text{gimpy} \), a mutant identified during the course of an ENU mutagenesis screen.

6.1 Rho family GTPases in the zebrafish

6.1.1 \( z\text{RhoA} \) and \( z\text{Racl} \) - the isolation of new family members

The screening of a late-somitogenesis stage zebrafish cDNA library led to the isolation of two zebrafish Rho homologues, subsequently named \( z\text{RhoA} \) and \( z\text{Racl} \) based on their sequence homology to those Rho family members cloned in other species. The expression pattern for both genes was found to be ubiquitous at early stages of development, becoming more restricted as embryogenesis proceeded. The probing of Northern blots for the presence of these transcripts confirmed the data shown by whole-mount \textit{in situ} analysis. Both of these did raise questions, still as yet unresolved, as to the apparent reduction of expression levels in the posterior regions of the body at later stages of development - could it be possible that \( \text{Rho} \) expression is downregulated? Are different isoforms of the genes being expressed? Could the stability and half-life of the proteins synthesised from the transcripts be adequate to maintain the requisite level of GTPase activity without the need for any further transcription? These questions are difficult to give satisfactory answers to. The development of functioning antisera to zebrafish RhoA and Rac1 would help to solve the perennial riddle of to what extent the transcripts seen by \textit{in situ} are converted into protein - currently, antibodies to chick RhoB (Liu & Jessell, 1998) and chick Rac1b (Malosio et al., 1997) are available, but neither of these gave satisfactory results when used in whole-mount immunohistology on zebrafish embryos. Indeed, Western blotting assays cast doubt on whether the particular RhoB epitope was present in zebrafish embryos at all.

There is scope for the further isolation of Rho and Rac genes in the zebrafish. The apparent genome duplication in the cyprinid lineage (Postlethwait et al., 1998) has led to the identification of additional members of many gene families (Amores et
6. General discussion

Evolutionary theory states that for duplicate genes to be preserved and remain functional, they must retain an essential function, and it has been found to be the case, for instance with the ephrin ligands, that an assumed 'founder' expression pattern from a single ancestral gene has been split into two domains, each controlled by one of the gene duplicates. Thus, as both expression domains are needed during development, there is selective pressure for the maintenance of both duplicates which become increasingly divergent as time passes. Indeed, it could even be argued that this theory explains the presence of a widely expressed Rho and a haemospecific Rac2, which is highly similar in sequence. Performing Southern blots on zebrafish genomic DNA would demonstrate the presence of any zRho or zRac duplicates, which could then be searched for using library screening as before. It would also be of interest to screen libraries of different embryonic stages and of different adult tissues, again to look for the presence of alternative Rho and Rac forms.

Ideally, the position of the cloned zRhoA and zRac1 will be determined on the zebrafish genetic map. It would be interesting to see whether they are candidate genes for any known mutations - however, it is possible that a mutation in either would disrupt embryogenesis so severely that embryos would never be recovered, particularly since many of the mutagenesis screens hitherto carried out do not look for abnormal phenotypes until 10hpf at the earliest.

6.1.2 Searching for other Rho family members

Looking for other Rho family members, such as Cdc42 or Mig-2, should prove relatively simple, given the advances forged in zebrafish genomics since the beginning of this project. The advent of the expressed sequence tag (EST) database has enabled researchers to search for their chosen gene of interest, using known or homologous sequences from other species, amongst a series of cloned fragments, a few hundred nucleotides of each having been sequenced. Once a gene of interest has been found, the cloned fragment of it is sent to the researcher, where it can be used in several ways: some choose to directly synthesise antisense RNA probes and perform in situ; alternatively, the fragment can be labelled and used to screen cDNA libraries at varying stringencies to isolate the full-length gene and others related to it; finally, some choose to use the EST sequence to design PCR primer pairs for their gene and amplify fragments of it from cDNA libraries themselves before using these to screen with. All of these would provide valid methods for the isolation of new and additional zebrafish GTPases and hopefully, once the EST database 'teething troubles' are resolved, this will be the case.
6. General discussion

6.1.3 Functional assays

As detailed in Chapter 4, some progress has been made in addressing the *in vivo* role of RhoA during early vertebrate development, with somewhat unexpected results. It seems that over-expression of the wild-type RhoA protein, or expression of a dominant negative form has no effect on embryogenesis. Although the reasons for this are not clear, it could be speculated that this is because there is little or no flux through the endogenous RhoA pathway at these early stages - however, this would require a slight leap of faith to explain the severe effects seen when all Rho function is ablated by C3 exoenzyme. Perhaps there is a specific RhoB or RhoC mediated pathway functioning at this time; or, more logically, the levels of functioning Rho present during early development are so high, with the presence of redundancy, that the exogenous levels of wild-type and dominant negative Rho protein which would be needed to show any effects cannot be achieved using RNA injection. This tallies with the dominant effects seen by the constitutively active form, which presumably is able to sequester factors away from the endogenous proteins and disrupt Rho-mediated signalling.

Had time not been such a pressing issue, similar constructs would have been generated for Rac1 and Cdc42 and the effects of their injection analysed. Although it is not possible to predict accurately the results of such experiments, it is tempting to speculate on their outcome, particularly where Rac1 is concerned. Rac1 has been reported to be required for the formation of three germ layers during gastrulation (Sugihara et al., 1998) and the effects on the modelling of the central nervous system and on the musculature would be of interest, since these are perturbed when Rac1 function is interfered with in *Drosophila* (Luo et al., 1994). A more finely-tuned approach would be to create DNA constructs in which the GTPase is under the control of a specific promoter and inject these into the early embryo - plans were afoot to put wild-type, dominant negative and constitutively active forms of Rac1 under the control of the GAP-43 (Reinhard et al., 1994) promoter alongside a GFP-actin construct. The injection of such would allow the examination of the effects of Rac1 on the growth of a particular subset of hindbrain neurons, for which the GAP-43 promoter is specific, with the GFP-actin allowing axon outgrowth and pathfinding decisions to be visualised in real-time using time-lapse confocal microscopy.

It would also be of interest to examine more precisely the roles these small GTPases may be playing during the early morphogenetic processes of
embryogenesis. Recent live work in the *Drosophila* embryo (Jacinto et al., 2000) has shown the existence of long, dynamic filopodia, dependent on Cdc42, which are required for the correct 'sensing' of matching cells across the divide during dorsal closure. Much as the patterns and general trends of cell movement during zebrafish embryogenesis are well-characterised, for example the dorsalward flow of cells during convergent extension, there has been no examination of the cells themselves. During epiboly, as cells proceed towards the vegetal pole, how are these movements brought about? Are there dynamic processes at the leading edge, tugging the cells vegetally? To what extent, if any, is there a loss of adhesion at the animal pole to facilitate the downward sweep? What role does the yolk cell play, does it form membrane ruffles on its surface similar to those of the exposed *Drosophila* amnioserosa? To address these questions, the mutant forms of the Rho GTPases could well provide an invaluable resource, coupled with high-magnification time-lapse confocal microscopy. After the situation in the wild-type embryo has been determined, using GFP-actin as a marker for actin-rich protrusions, the effects of interfering with Rho, Rac or Cdc42 could be examined, again by injecting RNA encoding the mutant forms. This could help to elucidate the precise role of these proteins during morphogenesis - however, this approach need not be restricted to the early stages of embryonic development. The formation of the somites is known to involve selective deadhesion between cells at the segment boundaries and so it could be speculated that this may require Rho GTPase-mediated process retraction. Alternatively, and perhaps having clinical relevance, the role of these proteins in the migration of organ primordia could be examined - for example, to what extent, if any, do the cardiac progenitors require Cdc42 to migrate to their correct final position? Are the GTPases needed to 'sense' a final settling point for such cells? The teaming up of GTPase-based reagents with video-microscopy is an exciting prospect for future work in zebrafish embryogenesis.

The cell autonomy of the effect produced with the constitutively active RhoA constructs could be assayed using transplantation experiments - taking injected, fluorescently labelled cells and placing them in a non-injected host or vice versa may give an idea of how the effects on epiboly are brought about; whether cells abutting injected neighbours behave normally or are 'sucked' into an abnormal stream of cell movements. Advances in live cell tracking technology should help to elucidate such matters in both injected and *silberblick* mutants - phenotypically, the same results are thought to be produced but whether the same group of cells is involved remains to be seen, as *silberblick* is thought to only exert its effect on a subset of anterior paraxial mesoderm cells (C.-P. Heisenberg, pers. comm.) but the injected construct has a more widespread expression than this.
Although the use of human forms of the Rho GTPases is generally accepted on account of the high level of amino acid conservation between even quite divergent species, it would still be interesting to look at the effects of injecting zebrafish GTPase-based constructs, cloning the full-length zRac1 as necessary, with the requisite substitutions engineered by site-directed mutagenesis to see if any subtleties in the resultant phenotype are seen. It would also be of interest to look at the effects of blocking GTPase function by way of morpholino injection, in which translation of the endogenous transcript is reputedly blocked by a helical formation between itself and the morpholino, an oligonucleotide corresponding to the 5' extreme of the transcript. This binds at the 5' extent and so blocks the translational start site in the majority of genes so far examined (Ekker, 2000; Nasevicius & Ekker, 2000).

Looking even further still into future directions this project could take, the prospect arises of the cloning of downstream pathway effectors such as Rho-kinase or POSH and looking at their expression patterns coupled with functional assays. Work has already been carried out on the effects of blocking Rho-kinase function using Y-27632 (Uchida et al., 2000) on epiboly and embryonic wound-healing in the zebrafish (K. Woolley, unpublished).

6.1.4 Summary

In all, the work presented here on the zebrafish Rho GTPase family is just the beginning of what could well contribute notable information into cellular behaviour during vertebrate embryogenesis, with a wide scope for further, fruitful investigation on this topic.
6. General discussion

6.2 Dwarf-like mutants in the zebrafish

6.2.1 Recovery of laminin mutations from mutagenesis screens

In the ENU mutagenesis screens previously carried out, the preponderance of alleles for the dwarf-like family of mutants was noteworthy, especially since for some genes only a single mutant allele has ever been isolated whereas presumably, mutant alleles have yet to be recovered for others. However, this may change with the advent of more specialised screens currently being carried out - it is hoped that genes required specifically for wound healing, as an example, may be isolated. This apparently unequal hit-rate for DNA mutagenesis by the ENU treatment may be due to differential packaging of the DNA, in that DNA which is more tightly packed or convoluted into a tertiary structure is less likely to be "hit". However, with respect to the dwarf mutants bashful, grumpy and sleepy/gimpy, a more plausible explanation for the excess of mutant alleles recovered (26 alleles have been recovered for bashful alone) is the size of the gene in which the mutation is being generated. Each laminin chain consists of several large exons, and so from a purely statistical point of view, it becomes more likely that deleterious mutations would be induced in these genes rather than in one which is somewhat shorter, for example RhoA.

The sheer size of the laminin chain transcripts entails that rescue experiments, in which mutant embryos are injected with the wild-type transcript to restore the function of the abnormal protein, are nigh-on impossible - the 6kb mRNA transcript for the laminin γ1 chain precludes the possibility of successful injection on account of its large size. However, morpholinos to the 5′ end of the γ1 chain have been used successfully in wild-type embryos to reproduce the sleepy/gimpy phenotype, confirming the genomic data as to the identity of the mutated gene (D. Stemple, unpublished).

As a similar project was being carried out simultaneously at NIMR, there is little supplementary work left to be performed in the characterisation of gimpy. Attempts have been made to assess the wound-healing capacity of 48hpf embryos, but initial results suggest that the fin buds are so badly held together that they disintegrate once punctured. Work is also underway to investigate the development of the circulatory system in mutant animals, as this is also severely affected by the absence of laminin. It remains to be seen whether this line can be developed as a clinical paradigm for human connective tissue disorders, but the potential is there for this mutant to be of great medical benefit.
6. General discussion

6.2.2 Summary

Although the process of mutagenesis screening the zebrafish is time-consuming and labour intensive, it can be thoroughly rewarding in terms of generating novel phenotypes. Coupled with advances in genetic mapping resources, it is now possible to determine the identity and location of the mutated gene in a relatively short space of time. The discovery of mutations in genes such as laminin, described here, provides inroads into using the zebrafish as a working model for human diseases and hopefully future screening will lead to the development of other models for processes such as wound healing and the development of the cardiovascular system.
CHAPTER SEVEN

Bibliography


7. Bibliography


240


7. Bibliography


247


7. Bibliography


Appendix 1

Full-length insert sequences

**ZRhoA (Clone23A11)**

CCGNTNCTCCCGCCTGCGGCGGCCGCTCTAGAACTAGTGGAGACCGCTGCNCTCCCGCCTGCGGCGGCCGCTCTAGAACTAGTGGAGA

**zRacl (Clone 23y)**

GTCGCACGAGCTGCTTGGCAGGAGAGAGAGAGAAGAGCTGCAATCTGAGCrCACCTGAGAGAAGAACAATGGGAAGCGGGGATGACGGGAAATGATATGG

262
TTACAATCTGCCTTTTTGTAGAGAATGAAATATTGCCAAAAGTTAAACCT
GCACTCAGTTTTGCTCACAATACGCTCTCGTTTTCTTGGAGACTTGGG
AAGGGGGGGGGGTTGTCAGAGGCGAGATTGCAGTAAACTGAAAGGAACTAT
TTTACAAGAGGAAAGTTGAAGAAACACCTTTTTTCCCCCGATGCTAG
AGACTTTTTTTTCTTTAAGTGTATAAAAGAAACCTAGTTAAATGCAT
ATAACAGCCTTTTGGTTCTCAGAGGTATAGCATATTCTCTACTACTGATCTG
AATTTAGGACTGACTCTGCTTAAAAACTCTCTGGTGTATTAGCTGCTAT
TGGAGATGTCCTGCGACGTGTGTTTATCCACTTTTTACAGTCCCAGTGTGTAT
TAGACTACAAGAACATCTCTACTGTATACTGATAACAGTAAATTGTTAGG
GTCATCAAGATTTAAAATCTCTAATTTGTACTTACTACACATGATCCGGTGGTT
GTGATTAGCCACAGATGGAGTGTCAATTTTGACAGTTAGAAATGACGATT
GAAATGTCTTATTTATTTGATCTTACACTAAATCTACAGTGT
ATTAACTTTTGTGCTAAAATACAAAAATGTAACAAAAAACAAAAA
AAAAAAAAACAGGGGGGGCCGNNAC
Appendix 2

'Strong' conservation groups
STA
NEQK
NHQK
NDEQ
QHRK
MILV
MILF
HY
FYW

'Weak' conservation groups
CSA
ATV
SAG
STNK
STPA
SGND
SNDEQK
NDEQHK
NEQHRK
FVLIM
HFY
Appendix 3

Sequences of primers used to amplify 300bp Rac fragments

5' BamHI GCGGGATTCCARGARGAYTAYGAYAG
3' EcoRI GGGGAATTCTGDGTNARDGCDGWRCAYTC
Appendix 4

Primer Sequences

hRho  5' CCGGAATTCCGCTGCCATCCGGGAAGAAA
       3' GCTCTAGAGCTCACAAGACAAGGCAACC

C3   5' CCGGAATTCCTATTCAAATACTTACCAG
       3' GCTCTAGAGCTTATTTAGGATTGATAGC

5' primers contain an EcoRI site; 3' XbaI.
List of Abbreviations

ADP adenosine diphosphate
ATP adenosine triphosphate
bp base-pairs
cDNA complementary DNA
CNS central nervous system
CTP cytidine triphosphate
DAB diaminobenzidine
DMSO dimethyl sulfoxide
DNA deoxyribonucleic acid
ECM extracellular matrix
EDTA ethylenediaminetetraacetic acid
ERM ezrin/radixin/moesin
ENU ethyl nitrosourea
GAP GTPase activating protein
GDI guanine nucleotide dissociation inhibitors
GDP guanosine diphosphate
GEF guanine nucleotide exchange factor
GTP guanosine triphosphate
H$_2$O$_2$ hydrogen peroxide
hpf hours post fertilisation
HRP horseradish peroxidase
JNK jun N-terminal kinase
kb kilobase
M molar
MAB maleic acid buffer
MAPK mitogen-activated protein kinase
mg milligram
mm millimetre
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>mM</td>
<td>millimolar</td>
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<tr>
<td>MBT</td>
<td>mid-blastula transition</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>magnesium chloride</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µm</td>
<td>micrometre</td>
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<tr>
<td>NaAc</td>
<td>sodium acetate</td>
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<tr>
<td>NaCl</td>
<td>sodium chloride</td>
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<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>NH₄Ac</td>
<td>ammonium acetate</td>
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<td>nm</td>
<td>nanometre</td>
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<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
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<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
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<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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<tr>
<td>PBS</td>
<td>phosho-buffered saline</td>
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<tr>
<td>PBT</td>
<td>phosho-buffered saline with 0.1% Tween-20</td>
</tr>
<tr>
<td>PBTx</td>
<td>phosho-buffered saline with 0.8% Triton-X-100</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>ribonucleic acid</td>
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<td>ribonuclease</td>
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<tr>
<td>RNAsin</td>
<td>ribonuclease inhibitor</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>saline sodium citrate buffer</td>
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<tr>
<td>SRF</td>
<td>serum response factor</td>
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<td>TAE</td>
<td>tris-acetate-EDTA buffer</td>
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<tr>
<td>TBE</td>
<td>tris-borate-EDTA buffer</td>
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<tr>
<td>TCA</td>
<td>tri-chloroacetic acid</td>
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<tr>
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<td>tris/EDTA buffer</td>
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<tr>
<td>TGFB</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine triphosphate</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>UV</td>
<td>ultra-violet</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>YSL</td>
<td>yolk synctial layer</td>
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