CHARACTERIZATION OF CREB-REGULATED TRANSCRIPTION COACTIVATOR 1 (crtc1) DURING EMBRYOGENESIS OF ZEBRAFISH (Danio rerio)

LAWRENCE SIE ENG KEAN

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to Nanyang Technological University in partial fulfillment of the requirement for the degree of Doctor of Philosophy

2014
This page intentionally left blank
ACKNOWLEDGEMENTS

I am most grateful to my supervisor Prof Mark Featherstone for his guidance, unwavering support and encouragement throughout my studies. I would like to thank members of my Thesis Advisory Committee, Prof Andrew Tan Nguan Soon and Prof Curt Alexander Davey for their time and invaluable inputs. I am truly grateful to School of Biological Science (SBS) NTU for the PhD scholarship award which made all this possible.

I would like to thank my lab members, Anusha, Ragini, Reshma, Ravi, Yvonne, Calista, Ser Yeng, Eva, Cyrus, Shen Hui, Siew Lee and Fiona for all their help, fruitful discussions, and encouragement.

LAWRENCE SIE
This page intentionally left blank
# TABLE OF CONTENTS

Contents

**ACKNOWLEDGEMENTS** .............................................................................................................i
**TABLE OF CONTENTS** ...........................................................................................................iii
**LIST OF FIGURES** ..................................................................................................................vii
**LIST OF TABLES** .....................................................................................................................ix
**LIST OF ABBREVIATIONS AND SYMBOLS** ........................................................................x
**ABSTRACT** ..........................................................................................................................xxi

## CHAPTER 1 : INTRODUCTION ...........................................................................................1

1.1. *Hox* genes .........................................................................................................................2

1.1.1. Clustered *Hox* genes ...................................................................................................2
1.1.2. *Hox* colinearity ..........................................................................................................4
1.1.3. HOX code .....................................................................................................................4
1.1.4. HOX protein domains ....................................................................................................5
  1.1.4.1. HOX Homeodomain ..............................................................................................5
  1.1.4.2. YPWM/ANW motif ...............................................................................................7
1.1.5. HOX monomeric DNA binding specificity ..................................................................9
1.1.6. Cooperative DNA binding with HOX cofactors ..........................................................9
1.1.7. Regulation of *Hox* expression ...................................................................................11
1.1.8. HOX function ................................................................................................................15

1.2.1. PBC class protein: PBX ..............................................................................................19
1.2.2. MEINOX class proteins: MEIS and PKNOX ..............................................................23
  1.2.2.1. *Pknox* genes .......................................................................................................23
  1.2.2.2. *Meis* genes ..........................................................................................................26
1.2.3. MEIS function ..............................................................................................................28

1.3. *Creb* genes .......................................................................................................................31

1.4. *Crtc* genes .........................................................................................................................37

1.5. *Crtc1* role in development .............................................................................................40
1.6. Zebrafish as a model organism .........................................................................................41

1.7. Morpholino knockdown .....................................................................................................42
1.8. Research objectives ...........................................................................................................43

## CHAPTER 2 : MATERIALS & METHODS .............................................................................45

2.1. Plasmid constructs ..............................................................................................................45
  2.1.1. Plasmids used in *in situ* hybridization (ISH) staining .............................................45
  2.1.2. Plasmids used in rescue experiment .........................................................................46
  2.1.3. Plasmid used in luciferase assay .................................................................................46
2.2. Chemicals, reagents, antibodies and zebrafish .................................................................47
2.2.1. Antibodies ................................................................................................ 47
2.2.2. DNA primers ........................................................................................... 47
2.2.3. Mammalian culture media ....................................................................... 48
2.2.4. Zebrafish strain ........................................................................................ 48

2.3. **ISH staining of zebrafish embryos** .............................................................. 48
2.3.1. Antisense labelled RNA probe synthesis ................................................. 48
2.3.2. Prevention of melanin pigmentation by phenylthiourea (PTU) .............. 49
2.3.3. Fixing embryos ........................................................................................ 49
2.3.4. ISH and washes ........................................................................................ 49
2.3.5. ISH and Immunodetection ....................................................................... 50

2.4. **Morpholino injection** .............................................................................. 51
2.4.1. Calibration of injection volume ............................................................... 51
2.4.2. Microinjection technique ......................................................................... 52
2.4.3. Morpholino design and order ................................................................... 53
2.4.4. Morpholino injection and rescue ............................................................. 53

2.5. **Staging of the zebrafish embryos** ............................................................ 54

2.6. **Immunodetection** .................................................................................. 54
2.6.1. Lysate preparation ................................................................................... 54
2.6.2. Immunoblot and immunodetection .......................................................... 55

2.7. **Real time quantitative polymerase chain reaction (PCR) amplification and detection** .................................................................................. 55
2.7.1. Total RNA isolation ................................................................................. 55
2.7.2. First strand cDNA synthesis .................................................................... 56
2.7.3. RT-qPCR primer design .......................................................................... 56
2.7.4. Real time reverse transcriptase-qPCR (RT-PCR) using SYBR Green .......................................................... 56

2.8. **Semi-quantitative RT-PCR** .................................................................... 56

2.9. **Luciferase reporter assay** ........................................................................ 57
2.9.1. Transfection of reporter and experimental DNA plasmids in cell culture .............................................................................................. 57
2.9.2. Luciferase reporter assay and detection ................................................... 57

2.10. **Electroporation** .................................................................................... 57

2.11. **Microscopy and imaging analysis** ......................................................... 58
2.12. **Statistical analysis** ................................................................................ 58
2.13. **Zebrafish handling and care** ................................................................ 58

**CHAPTER 3: RESULTS** .................................................................................. 59
3.1. **Spatial and temporal expression of zebrafish crtl and crtc1b during early embryonic development** ........................................................................ 59
3.2. Characterization of zebrafish Crtc1 and Crtc1b ........................................ 60
3.3. Colocalization of crtc1 (and crtc1b) transcript expression with meis1 (nm_131893) in 24 hpf zebrafish embryo ......................................................... 65
3.4. Morpholino mediated knockdown of crtc1 function ...................................... 71
   3.4.1. Splice blockers targeting crtc1 and crtc1b disrupt splicing and have pleiotropic effects on zebrafish development ......................................... 71
   3.4.2. Translation blocker ................................................................................. 77
   3.4.2.1. crtc1 tMO1 reduced Crtc1-flag expression in electroporated HEK293T cells ........................................................................................................ 77
   3.4.2.2. crtc1 tMO1 morphant phenotype ............................................................ 80
   3.4.2.3. Apoptosis in crtc1 morphants ............................................................... 83
   3.4.2.4. The crtc1 morphant phenotype is independent of p53 function .................. 83
   3.4.2.5. An independent and non-overlapping morpholino, crtc1 tMO2, elicited a similar phenotype .............................................................. 85
   3.4.2.6. Curly-tail-down phenotype was partially rescued with RNA expressing Crtc1-flag ................................................................................. 85
   3.4.3. Focus on translation morphants ............................................................... 88
3.5. In situ hybridization analysis ........................................................................ 89
   3.5.1. Eye development is affected in crtc1 morphants ........................................ 89
   3.5.2. Brain growth/development affected in crtc1 morphants ............................. 99
   3.5.3. Other phenotypes ................................................................................... 107
   3.5.3.1. Dorsoventral (DV) patterning at gastrula stage affected in crtc1 morphant ........................................................................................................ 107
   3.5.3.2. Mild deviation of heart left-right (LR) asymmetry in morphant ............... 110
   3.5.3.3. Convergent movement affected in morphants ........................................ 113
   3.5.3.4. Neural crest migration and terminal nerve (TN) development is affected in crtc1 morphants .............................................................. 114
   3.5.3.5. DV patterning in retina affected in morphant ........................................ 118
   3.5.3.6. Myelination of neurons might be affected in morphants ....................... 120

CHAPTER 4 : DISCUSSION ............................................................................ 122
4.1. Spatiotemporal expression of crtc1 members during embryonic and early larval development ................................................................. 122
4.2. Coactivator activity of Crtc1 and Crtc1b ...................................................... 123
4.3. Differential post-translational modification of Crtc1b isoforms ....................... 124
4.4. Colocalization of meis1 and crtc1 expression ............................................... 125
4.5. Distinct effect of tMO- and splMO-mediated knock down of crtc1 .................. 125
4.6. Partial functional redundancy of Crtc1 members ......................................... 126
4.7. crtc1 morphants phenotype ....................................................................... 127
4.8. Midline structure in crtc1 morphants .......................................................... 128
4.9. Brain and eye development in crtc1 morphants ........................................ 130
4.10. CRTC1 in cell survival, proliferation and apoptosis .............................. 132
4.11. CRTC1 in other embryonic processes ....................................................... 136
CHAPTER 5 : CONCLUSION & FUTURE DIRECTIONS ....................................... 137
APPENDIX ............................................................................................................ 141
1. Multiple amino acid sequence alignment of zebrafish Crtc1 and Crtc1b with CRTC1 subfamily members from other organisms ........................................ 141
2. Multiple amino acid sequence alignment showing exon 11 of Crtc1b aligned with comparable region of other CRTC1s (e.g. Crtc2 and Crtc3) of human and mouse. ................................................... 144
3. Dose response of crtc1b tMO1 ................................................................... 145
4. List of DNA constructs used in the study ...................................................... 146
   pST Meis2b ......................................................................................................... 146
   pST zGsc ............................................................................................................. 147
   p4T cyp26a1 ....................................................................................................... 148
   pSP zCRTC1-flag ............................................................................................. 149
   pSP RzCRTC1-flag ........................................................................................... 150
   pcDNA3.1 Crtc1 ............................................................................................... 151
   pcDNA3.1 Crtc1-flag ........................................................................................ 152
   pcDNA3.1 early Crtc1b .................................................................................... 153
   pcDNA3.1 late Crtc1b ....................................................................................... 154
   pCS2+ Crtc1-flag ............................................................................................. 155
   pCS2+ vector ..................................................................................................... 156
   pST vector ........................................................................................................... 157
5. Sense control for WISH of crtc1 and crtc1b expression in zebrafish embryos ........................................................................................................ 158
6. Dynamic expression of 18S rRNA during zebrafish development .............. 160
7. p53 and delta113 p53 expression in controls and crtc1 morphants .............. 161
8. Gene and protein nomenclature guideline ................................................... 162
REFERENCES ...................................................................................................... 163
**LIST OF FIGURES**

| Figure 1.1 | Hox cluster duplication and gene loss through evolutionary time. | 3 |
| Figure 1.2 | Spatial colinear expression of murine Hox genes along the embryonic vertebral column. | 6 |
| Figure 1.3 | Primary, secondary and tertiary structure of the HD. | 8 |
| Figure 1.4 | Hindbrain HOX code. | 18 |
| Figure 1.5 | The structure of mouse Creb gene. | 33 |
| Figure 3.1 | Spatio-temporal expression of crtl during early zebrafish development. | 61 |
| Figure 3.2 | Spatio-temporal expression of crtlb during early zebrafish development. | 62 |
| Figure 3.3 | Semi-quantitative PCR assessment of crtl and crtlb transcript expression level during early zebrafish development. | 63 |
| Figure 3.4 | Phylogenetic tree of CRTCs genes. | 64 |
| Figure 3.5 | Crcl coactivator activity in CRE-luciferase reporter assay. | 66 |
| Figure 3.6 | Crcl coactivator activity in CRE-luciferase reporter assay. | 66 |
| Figure 3.7 | Spatiotemporal expression of meisl during zebrafish development. | 68 |
| Figure 3.8 | Overlap region of crtl and crtlb with meisl expression deduced from in situ hybridization staining. | 69 |
| Figure 3.9 | Colocation of crtl and meisl transcript expression domains in 24 hpf zebrafish embryo. | 70 |
| Figure 3.10 | Splice morpholinos caused altered splicing of crtl. | 73 |
| Figure 3.11 | Splice MOs caused altered splicing of crtlb. | 74 |
| Figure 3.12 | Phenotype changes in crtl splice morphants at different doses of MO. | 76 |
| Figure 3.13 | Phenotype changes in crtlb splice morphants at different doses of MO. | 76 |
| Figure 3.14 | Translation blocker (tMO1) reduced expression of Crtc1-flag in HEK293T cells. | 79 |
| Figure 3.15 | Translation blocker (tMO1) reduced the expression of Crtc1-flag in 24 hpf zebrafish embryo. | 79 |
| Figure 3.16 | Dose response of crtl tMO1. | 81 |
| Figure 3.17 | Phenotype of crtl tMO1 morphants. | 82 |
| Figure 3.18 | Apoptosis induced by MO in crtl morphant. | 84 |
| Figure 3.19 | Comparable curly-tail-down phenotype in non-overlapping tMO2 injected morphants. | 86 |
| Figure 3.20 | Capped and polyadenylated RNA expressing Crtc1-flag and “MO-resistant” Crtc1-flag in HEK293T cells. | 87 |
| Figure 3.21 | RNA expressing tMO1-resistant Crtc1-flag partially rescued the mutant phenotype of tMO1 morphant. | 88 |
| Figure 3.22 | isl1 expression in 24 hpf morphant and control embryo in wholemount and flatmount in situ hybridization (ISH) stain. | 90 |
| Figure 3.23 | isl1 expression in 48 hpf morphant and control embryo in wholemount ISH stain. | 91 |
| Figure 3.24 | neurod4 expression in 24 hpf morphants and control embryos in wholemount ISH stain. | 93 |
Figure 3.25  *neurod4* expression in 48 hpf morphant and control embryos in wholemount ISH stain ................................................................. 93

Figure 3.26  *neurod4* expression in 24 and 48 hpf morphants co-injected with *p53* tMO and un.injected control embryos in wholemount ISH ........................................................................ 94

Figure 3.27  *olig2* expression in 24 hpf morphant and control in wholemount and flatmount ISH ........................................................... 95

Figure 3.28  *olig2* expression in 48 hpf morphant and control in wholemount ISH ............................................................................... 96

Figure 3.29  *meis2b* expression in 24 hpf control and morphant (co-injected with *p53* tMO) in wholemount and flatmount ISH ............ 97

Figure 3.30  *meis2b* expression in 48 hpf control and morphant (co-injected with *p53* tMO) in wholemount ISH ........................................ 97

Figure 3.31  *meis1* expression in 24 hpf control and morphant in wholemount and flatmount ISH ....................................................... 98

Figure 3.32  *meis1* expression in 48 hpf control and morphant in wholemount ISH ............................................................................... 99

Figure 3.33  *ascl1b* expression in 24 hpf control, morphant, and rescued embryos in wholemount ISH .................................................. 100

Figure 3.34  *ascl1b* expression in 24 hpf control and morphants (both co-injected with *p53* tMO) in wholemount and flatmount ISH ................................................................. 100

Figure 3.35  *ascl1b* expression in 48 hpf control, morphant, rescued embryos in wholemount ISH ....................................................... 102

Figure 3.36  *ascl1b* expression in 48 hpf control and morphant (both co-injected with *p53* tMO) in wholemount ISH ................................. 103

Figure 3.37  *nkx2.2a* expression in 24 hpf control, morphant and rescued embryos in wholemount ISH .................................................. 104

Figure 3.38  *nkx2.2a* expression in 48 hpf control and morphant (co-injected with *p53* tMO) in wholemount ISH ................................. 104

Figure 3.39  *nkx2.2a* expression in 48 hpf control, morphant and rescued embryos in wholemount ISH .................................................. 105

Figure 3.40  *nkx2.2a* expression in 48 hpf control and morphant (co-injected with *p53* tMO) in wholemount ISH ................................. 106

Figure 3.41  Measurement of brain size by correlation to width of *ascl1b* and *nkx2.2a* expression domains ........................................... 107

Figure 3.42  *bmp4* expression in shield stage (6 hpf) controls, morphants and rescued embryos (co-injected with *p53* tMO) in wholemount ISH ............................................................................. 108

Figure 3.43  *gsc* expression in shield stage (6 hpf) controls, morphants and rescued embryos (co-injected with *p53* tMO) in wholemount ISH ............................................................................. 109

Figure 3.44  Heart jogging assessed by *bmp4* expression in heart primordium in controls and morphants ................................................................. 111

Figure 3.45  Otolith number in controls and morphants ......................................................................................................................... 112

Figure 3.46  Convergence movement assessed by width of *myod1*-expressing cells of the lateral mesoderm ......................................................... 114

Figure 3.47  *sox10*-expression in 24 hpf and 48 hpf control and morphant embryos in wholemount ISH .......................................................... 116

Figure 3.48  *sox10*-expression in 24 hpf controls and morphants (co-injected with *p53* tMO) in wholemount ISH .................................................. 117
Figure 3.49  *sox10*-expression in 48 hpf controls and morphants (co-injected with p53 tMO) in wholemount ISH ............................................................. 117

Figure 3.50  *cyp26a1* expression in controls and morphants (co-injected with p53) in wholemount ISH ........................................................................ 119

Figure 3.51  *plp1b* expression in controls and morphants in wholemount ISH ................................................................................................. 121

Figure 4.1  CRTC1 role in cell proliferation, survival, apoptosis, and differentiation .......................................................................................... 135

**LIST OF TABLES**

Table 2.1  List of primer sequence used in semi-quantitative RT-PCR .......... 47

Table 2.2  List of primer sequence used in quantitative RT-PCR ................. 47

Table 2.3  Duration of 10 µg/ml proteinase K permeabilization in relation to the developmental stage of embryos ........................................ 50

Table 2.4  Relationship between injection volume and diameter of spherical volume ..................................................................................... 52

Table 2.5  List of translation and splice morpholinos and their corresponding sequences ................................................................. 53

Table 2.6  Developmental period represented by different stages of embryonic development ................................................................. 54
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-3-3 protein</td>
<td>14-3-3 family members. Also known as Tyrosine 3-Monoxygenase/Tryptophan 5-Monoxygenase Activation Protein (YWHA)</td>
</tr>
<tr>
<td>abd-A</td>
<td>abdominal A gene in fly; it is evolutionary close to HOX genes in the 8th paralog group</td>
</tr>
<tr>
<td>Abd-B</td>
<td>Abdominal B gene in fly; it is evolutionary close to HOX genes in paralog groups 9 to 13 (or 14 in amphioxus).</td>
</tr>
<tr>
<td>AC</td>
<td>amacrine cell</td>
</tr>
<tr>
<td>ACREB</td>
<td>acidic CREB is a dominant negative form of CREB protein</td>
</tr>
<tr>
<td>actb</td>
<td>actin, beta gene</td>
</tr>
<tr>
<td>AF6 (MLLT4)</td>
<td>Myeloid/Lymphoid Or Mixed-Lineage Leukemia (Trithorax Homolog, Drosophila); Translocated To, 4</td>
</tr>
<tr>
<td>AGM</td>
<td>aorta-gonad-mesonephros, a region in which definitive HSCs arise</td>
</tr>
<tr>
<td>aka</td>
<td>also known as</td>
</tr>
<tr>
<td>AKT</td>
<td>V-Akt murine thymoma viral oncogene homolog gene family</td>
</tr>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase heterotrimeric complex consisting of an alpha catalytic subunit, and non-catalytic beta and gamma subunits</td>
</tr>
<tr>
<td>ANT-C</td>
<td>Antennapedia Complex (ANT-C or ANTC) gene family (or gene &quot;complex&quot;); This family closely resembles HOX genes from paralog groups 1 to 6 and encodes homeodomain containing transcription factors.</td>
</tr>
<tr>
<td>Antp</td>
<td>Antennapedia gene in fly; it is evolutionary close to HOX in 6th paralog group</td>
</tr>
<tr>
<td>ANW</td>
<td>Ala-Asn-Trp motif situated at the N-terminal arm of homeodomain of HOX protein (in paralog groups 9 and 10) which is important for interaction with PBC HD protein; this motif is very similar to YPWM/FDWM found in HOX in paralog groups 1 to 8</td>
</tr>
<tr>
<td>AP</td>
<td>anterior posterior axis</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activating protein 1 (AP-1) is a protein complex</td>
</tr>
<tr>
<td>ARE</td>
<td>auto-regulatory cis-element</td>
</tr>
<tr>
<td>ascl1b</td>
<td>achaete-scute family bHLH transcription factor 1b; in zebrafish there are two genes, ascl1a and ascl1b arising from gene duplication</td>
</tr>
<tr>
<td>ATF</td>
<td>activating transcription factor (ATF) subfamily are member of bZIP (basic-region leucine zipper) transcription factor family.</td>
</tr>
<tr>
<td>ATF2</td>
<td>activating transcription factor 2 (formerly known as CREB2)</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia-telangiectasia mutated protein</td>
</tr>
<tr>
<td>BA2</td>
<td>second branchial arch</td>
</tr>
<tr>
<td>bcd</td>
<td>bicoid gene</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell CLL/lymphoma 2 (or BCL2) protein is an integral outer mitochondrial membrane protein that is anti-apoptotic.</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------</td>
<td>------------</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix loop helix structural motif</td>
</tr>
<tr>
<td>bhlhe23</td>
<td>basic helix-loop-helix family, member e23; bhlhe23 (BHLHE23 in human) encodes a member of the bHLH transcription factor family</td>
</tr>
<tr>
<td>bmp4</td>
<td>bone morphogenetic protein 4; bmp4 (or BMP4 in human) encodes protein belonging to transforming growth factor-beta superfamily</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BP</td>
<td>bipolar cell</td>
</tr>
<tr>
<td>BX-C</td>
<td>Bithorax Complex (BX-C) gene family (or gene &quot;complex&quot;); This family closely resembles HOX genes from paralog groups 7 to 13 and encodes homeodomain containing transcription factors.</td>
</tr>
<tr>
<td>BXH-2</td>
<td>BXH-2 or BXH2 describes laboratory mouse strain (visit the Jackson laboratory at <a href="http://jaxmice.jax.org/strain/000034.html">http://jaxmice.jax.org/strain/000034.html</a>)</td>
</tr>
<tr>
<td>bZIP</td>
<td>basic region leucine zipper domain</td>
</tr>
<tr>
<td>C1</td>
<td>cervical segment 1</td>
</tr>
<tr>
<td>CaMK</td>
<td>Calcium/calmodulin dependent protein kinase</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate is a second messenger important in many biological processes</td>
</tr>
<tr>
<td>CaN</td>
<td>Calcineurin protein complex consisting of regulatory subunit and a catalytic subunit</td>
</tr>
<tr>
<td>Cartpt</td>
<td>cocaine and amphetamine–regulated transcript (CART) prepropeptide</td>
</tr>
<tr>
<td>CBP</td>
<td>Creb binding protein (also known as CREBBP abbreviation)</td>
</tr>
<tr>
<td>CBX</td>
<td>Chromobox Homolog</td>
</tr>
<tr>
<td>ccna1</td>
<td>cyclin A1</td>
</tr>
<tr>
<td>ccnd</td>
<td>cyclin D</td>
</tr>
<tr>
<td>ccnd1</td>
<td>cyclin D1</td>
</tr>
<tr>
<td>ccnd3</td>
<td>cyclin D3</td>
</tr>
<tr>
<td>ccne1</td>
<td>cyclin E1</td>
</tr>
<tr>
<td>CD41</td>
<td>also known as Integrin, Alpha 2b (Platelet Glycoprotein IIb Of IIb/IIIa Complex, Antigen CD41) with acronym ITGA2B; it encodes integrin alpha chain 2b, which have been used as cell surface marker in immunophenotyping blood cells</td>
</tr>
<tr>
<td>CDX</td>
<td>Caudal type homeobox gene family;</td>
</tr>
<tr>
<td>CE</td>
<td>convergent extension</td>
</tr>
<tr>
<td>Ceh-20</td>
<td>This gene is exd ortholog (or PBX in human)</td>
</tr>
<tr>
<td>c-fos (fos)</td>
<td>also known as FBJ Murine Osteosarcoma Viral Oncogene Homolog (abbreviated fos); it encodes a member of bZIP gene family of transcription factors</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>c-jun (jun)</td>
<td>also known as jun proto-oncogene (abbreviated jun); it encodes a member of bZIP gene family of transcription factors</td>
</tr>
<tr>
<td>CK</td>
<td>casein kinase</td>
</tr>
<tr>
<td>c-myc (myc)</td>
<td>V-Myc Avian Myelocytomatosis Viral Oncogene Homolog (abbreviated myc); it encodes bHLH transcription factor</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response cis-element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP responsive element binding protein; CREB gene family encodes bZIP family of transcription factors involved in cAMP-PKA signaling, among others.</td>
</tr>
<tr>
<td>Creb3l2</td>
<td>CAMP Responsive Element Binding Protein 3-Like 2; Creb3l2 encodes a member of the oasis bZIP transcription factor family</td>
</tr>
<tr>
<td>CREBM1</td>
<td>CREBM1 is a inactive CREB, with non-phosphorylatable Ser133Ala point mutation.</td>
</tr>
<tr>
<td>CREM</td>
<td>cAMP responsive element modulator; this gene encodes bZIP transcription factor</td>
</tr>
<tr>
<td>crh-1</td>
<td>Creb ortholog in C. elegans</td>
</tr>
<tr>
<td>CRISPR</td>
<td>clustered regularly interspaced short sequences (or CRISPR) is part of CRISPR/Cas system for prokaryotic immunity against foreign genetic elements; it has been developed as a targeted mutagenesis technique</td>
</tr>
<tr>
<td>CRTC</td>
<td>CREB Regulated Transcription Coactivator; CRTC gene family encodes positive coregulators for CREB, AP-1 and MEIS transcription factors; CRTC member, e.g. CRTCl is known previously as MECT1/TORC1</td>
</tr>
<tr>
<td>crtc1</td>
<td>CREB regulated transcription coactivator 1 (NCBI accession no. XM_003199425);</td>
</tr>
<tr>
<td>crtc1b</td>
<td>CREB regulated transcription coactivator 1b (NCBI accession no. NM_001077457);</td>
</tr>
<tr>
<td>excr4b</td>
<td>chemokine (C-X-C motif), receptor 4b; it encodes a 7-pass transmembrane G-protein coupled receptor involved in regulating intracellular calcium level and MAPK1/MAPK3 activation; it is also used as RGC marker</td>
</tr>
<tr>
<td>cyp26a1</td>
<td>cytochrome P450, family 26, subfamily A, polypeptide 1; it encodes a member of the cytochrome P450 superfamily of enzymes and is involved in retinoic acid (RA) catabolism, and hence regulate RA level</td>
</tr>
<tr>
<td>Dfd</td>
<td>Deformed (D. melanogaster); it belongs to ANT-C homeobox gene family; it is evolutionary close to HOX in 4th paralog group</td>
</tr>
<tr>
<td>dpc</td>
<td>days post coitus in mouse development</td>
</tr>
<tr>
<td>dpf</td>
<td>days post fertilization in zebrafish development</td>
</tr>
<tr>
<td>DV</td>
<td>dorsal ventral axis</td>
</tr>
<tr>
<td>E2A</td>
<td>also known as Transcription Factor 3 (TCF3 in human); it encodes a member of the E protein (class I) family of bHLH transcription factors</td>
</tr>
<tr>
<td>EED</td>
<td>embryonic ectoderm development; This gene encodes a member of the Polycomb-group (PcG) family (encoding subunits involved in PcG complex function), which has protein scaffold function for formation of PRC2/EED-EZH2 complex</td>
</tr>
<tr>
<td>eef1a1lll</td>
<td>eukaryotic translation elongation factor 1 alpha 1, like 1 (NM_131263)</td>
</tr>
<tr>
<td>efn</td>
<td>ephrin ligands</td>
</tr>
<tr>
<td>EGF2</td>
<td>early growth response 2 (aka KROX20)</td>
</tr>
<tr>
<td>ENL</td>
<td>also known as myeloid/lymphoid or mixed-lineage leukemia (Trithorax homolog, Drosophila); translocated to, 1 [MLLT1]),</td>
</tr>
</tbody>
</table>
which is a component of the super elongation complex (SEC) required for processivity of RNA pol II. ENL is fused to MLL in leukemia.

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENU</td>
<td>N-ethyl-N-nitrosourea which is a mutagen used in zebrafish (and other model organisms) to target spermatogonial stem cells by introducing point mutations</td>
</tr>
<tr>
<td>eph</td>
<td>ephrin receptors</td>
</tr>
<tr>
<td>ephb2</td>
<td>EPH receptor B2</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>Ex11_domain</td>
<td>exonic region alternatively spliced into late Crtc1 protein</td>
</tr>
<tr>
<td>exd</td>
<td>extradenticle; exd encodes a TALE-HD protein, closely related to PBC protein family</td>
</tr>
<tr>
<td>exportin</td>
<td>exportin belongs to karyopherins, which are family of proteins involved in trafficking molecules to and fro cytoplasm and nucleus; karyopherins can be classified as importins (into nucleus), exportins (out of nucleus) or both (with dual function) based on the direction of transport; CRM1 is an example of exportin</td>
</tr>
<tr>
<td>EZH1</td>
<td>enhancer of zeste homolog 1 (Drosophila) is a subunit of Polycomb repressive complex-2 (PRC2) that mediates methylation of histone H3</td>
</tr>
<tr>
<td>EZH2</td>
<td>enhancer of zeste homolog 2 (Drosophila) is a subunit of Polycomb repressive complex-2 (PRC2) that mediates methylation of histone H3</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>Fgf3</td>
<td>fibroblast growth factor 3</td>
</tr>
<tr>
<td>FLT3</td>
<td>fms-related tyrosine kinase 3</td>
</tr>
<tr>
<td>foxd1</td>
<td>forkhead box D1</td>
</tr>
<tr>
<td>G6PC</td>
<td>glucose-6-phosphatase, catalytic subunit</td>
</tr>
<tr>
<td>GDX</td>
<td>gene expression database team at Jackson Laboratory (<a href="http://www.informatics.jax.org/">http://www.informatics.jax.org/</a>)</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GLC</td>
<td>ganglionic cell layer</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-Acetylglicosamine</td>
</tr>
<tr>
<td>GnRH3</td>
<td>gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>GOI</td>
<td>gene of interest</td>
</tr>
<tr>
<td>gsc</td>
<td>goosecoid</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyltransferase</td>
</tr>
<tr>
<td>HCNRs</td>
<td>highly conserved non-coding regions in the genome sequence</td>
</tr>
<tr>
<td>HD</td>
<td>homeodomain</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HES-1</td>
<td>hairy and enhancer of split 1, (Drosophila) is a bHLH transcription factor (repressor)</td>
</tr>
<tr>
<td>Hh</td>
<td>hedgehog</td>
</tr>
<tr>
<td>HH stage</td>
<td>Hamburger–Hamilton staging in chick (refers H&amp; H, 1992)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>hMRP8</td>
<td>human Multidrug Resistance-Associated Protein 8 (also known as ATP-binding cassette, subfamily C [CFTR/MRP], member 11; or ABCC11)</td>
</tr>
<tr>
<td>HOX</td>
<td>Homeobox gene family termed HOX; there are a total of 39 HOX genes divided into 4 clusters in vertebrates (see Fig. 1.1), e.g. HOXD4 denotes HOX gene in D cluster at 4th paralog group.</td>
</tr>
<tr>
<td>hpf</td>
<td>hours post fertilization in zebrafish development</td>
</tr>
<tr>
<td>HR1 (or MH1)</td>
<td>MEINOX protein family subdomain</td>
</tr>
<tr>
<td>HR2 (or MH2)</td>
<td>MEINOX protein family subdomain</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
</tr>
<tr>
<td>HTH</td>
<td>homothorax</td>
</tr>
<tr>
<td>ift88</td>
<td>intraflagellar transport 88 homolog (Chlamydomonas)</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>Igf1</td>
<td>insulin-like growth factor 1 (somatomedin C)</td>
</tr>
<tr>
<td>ihhhb</td>
<td>Indian hedgehog homolog b</td>
</tr>
<tr>
<td>IL8</td>
<td>interleukin 8</td>
</tr>
<tr>
<td>Ile-47</td>
<td>Amino acid residue Isoleucine at position 47 of the polypeptide (or protein) sequence-in-question, e.g. 60 aa HD sequence of HOX proteins.</td>
</tr>
<tr>
<td>importin</td>
<td>importin belongs to karyopherins, which are family of proteins involved in trafficking molecules to and fro cytoplasm and nucleus; karyopherins can be classified as importins (into nucleus), exportins (out of nucleus) or both (with dual function) based on the direction of transport; karyopherin (KPN) is an example of importin</td>
</tr>
<tr>
<td>INL</td>
<td>inner nuclear layer</td>
</tr>
<tr>
<td>IRO</td>
<td>Iroquois Homeobox protein family (also known as IRX) involved in embryonic patterning</td>
</tr>
<tr>
<td>irx6a</td>
<td>Iroquois Homeobox 6a gene in zebrafish</td>
</tr>
<tr>
<td>ISH</td>
<td>in situ hybridization</td>
</tr>
<tr>
<td>islet1</td>
<td>ISL LIM homeobox 1 gene</td>
</tr>
<tr>
<td>JNK</td>
<td>JNK1; also known as Mitogen-Activated Protein Kinase 8 (MAPK8)</td>
</tr>
<tr>
<td>KID</td>
<td>kinase inducible domain of CREB protein</td>
</tr>
<tr>
<td>Kiss</td>
<td>KiSS-1 metastasis-suppressor</td>
</tr>
<tr>
<td>KIX</td>
<td>KID-interacting (KIX) protein domain of CREB protein</td>
</tr>
<tr>
<td>KLF</td>
<td>Kruppel-like factors</td>
</tr>
<tr>
<td>KMT2A</td>
<td>lysine (K)-specific methyltransferase 2A</td>
</tr>
<tr>
<td>lab</td>
<td>labial gene belongs to Antp homeobox family; it is evolutionary close to HOX in 1st paralog group</td>
</tr>
<tr>
<td>lazarus</td>
<td>pbx4 gene in zebrafish</td>
</tr>
<tr>
<td>lhxl1a</td>
<td>LIM homeobox 1 gene in zebrafish</td>
</tr>
<tr>
<td>Lin</td>
<td>lineage markers used for immunophenotyping cells which are used specifically for identifying mature blood cells and comprise a set of &quot;Lin&quot; antigens CD2, CD3, CD4, CD5, CD8, NK1.1, B220, TER-119, Gr-1; cells that lack/low of these markers are known as Lin(-), and vice versa for Lin(+).</td>
</tr>
<tr>
<td><strong>lmo2</strong></td>
<td>LIM domain only 2 (rhombotin-like 1) gene</td>
</tr>
<tr>
<td>----------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>LR</td>
<td>left-right axis</td>
</tr>
<tr>
<td>LTP</td>
<td>long term potentiation of synaptic activity</td>
</tr>
<tr>
<td>Ly5.1</td>
<td>lymphocyte antigen Ly5.1 is a surface marker corresponding to cluster of differentiation 45.1 (CD45.1) which is believed to be identical to Ly5.2 (CD45.2) and have been grouped collectively as CD45, which is a white blood cell common antigen</td>
</tr>
<tr>
<td>Ly6a</td>
<td>lymphocyte antigen 6 complex, locus A (or ly6a) is used as a surface marker for stem cell (or multipotent HSC) antigen, which is similar to Sca-1.</td>
</tr>
<tr>
<td>MAFB</td>
<td>v-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog B (aka KREISLER or KRML)</td>
</tr>
<tr>
<td>MAML2</td>
<td>mastermind-like 2 (Drosophila) is a transcriptional coactivator for NOTCH proteins</td>
</tr>
<tr>
<td>MAPKAPK</td>
<td>mitogen-activated protein kinase-activated protein kinase</td>
</tr>
<tr>
<td>MBT</td>
<td>mid-blastula transition</td>
</tr>
<tr>
<td>MEINOX</td>
<td>Meis and Prep/Pknox (MEINOX family) proteins</td>
</tr>
<tr>
<td>MEIS</td>
<td>Myeloid Ecotropic Viral Integration Site homolog (gene name derived from mouse study); MEIS gene family belongs to homeobox gene family termed MEINOX (or MEIS/PKNOX; portmanteau of MEIS and PKNOX) and encodes for MEINOX domain containing transcription factor.</td>
</tr>
<tr>
<td>MEIS1</td>
<td>Meis homeobox 1 protein</td>
</tr>
<tr>
<td>meis1</td>
<td>Meis homeobox 1 gene</td>
</tr>
<tr>
<td>MEIS1A</td>
<td>Meis homeobox 1A protein isoform with transactivation domain (TAD)</td>
</tr>
<tr>
<td>meis2b</td>
<td>Meis homeobox 2b gene in zebrafish; there are two meis2 genes in zebrafish due to gene duplication</td>
</tr>
<tr>
<td>MG</td>
<td>Muller glia</td>
</tr>
<tr>
<td>MLL</td>
<td>mixed lineage leukemia; the protein is also known as MLL (also known as KMT2A)</td>
</tr>
<tr>
<td>MO</td>
<td>morpholino antisense oligo (or just morpholino)</td>
</tr>
<tr>
<td>MOHAWK(MK X)</td>
<td>mohawk homeobox protein family</td>
</tr>
<tr>
<td>MSK</td>
<td>mitogen- and stress-activated kinases</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>Myf5</td>
<td>myogenic factor 5 protein</td>
</tr>
<tr>
<td>Myod1</td>
<td>myogenic differentiation 1 protein. Previously known as MyoD.</td>
</tr>
<tr>
<td>NES</td>
<td>nuclear export signal protein domain/motif</td>
</tr>
<tr>
<td>neurod4</td>
<td>neuronal differentiation 4 gene</td>
</tr>
<tr>
<td>NF stage</td>
<td>Nieuwkoop and Faber staging of Xenopus embryonic/larval stage (refers <a href="http://www.xenbase.org/anatomy/alldev.do">http://www.xenbase.org/anatomy/alldev.do</a>)</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor (beta polypeptide)</td>
</tr>
<tr>
<td>nkx2.2a</td>
<td>NK2 homeobox 2a gene in zebrafish</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal protein domain/motif</td>
</tr>
<tr>
<td>Nodal</td>
<td>Nodal Growth Differentiation Factor is a morphogen involved in TGF-beta signaling</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------</td>
<td>------------</td>
</tr>
<tr>
<td>Nr5a1</td>
<td>Nuclear receptor subfamily 5, group A, member 1 gene (aka Ad4bp or SF-1)</td>
</tr>
<tr>
<td>NT</td>
<td>nasal temporal axis</td>
</tr>
<tr>
<td>NTF</td>
<td>neurotrophin</td>
</tr>
<tr>
<td>OGT</td>
<td>O-linked GlcNAc transferase</td>
</tr>
<tr>
<td>olig2</td>
<td>oligodendrocyte lineage transcription factor 2 gene</td>
</tr>
<tr>
<td>ONBL</td>
<td>outer neuroblastic layer</td>
</tr>
<tr>
<td>OVL</td>
<td>otic vesicle length (measurement to determine embryo developmental stage)</td>
</tr>
<tr>
<td>p15</td>
<td>also known as cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4) or Cdkn2b</td>
</tr>
<tr>
<td>p16</td>
<td>also known as Cyclin-Dependent Kinase Inhibitor 2A or Cdkn2a</td>
</tr>
<tr>
<td>p21</td>
<td>also known as Cyclin-Dependent Kinase Inhibitor 1A (p21, Cip1) or Cdkn1a</td>
</tr>
<tr>
<td>P300</td>
<td>also known as E1A binding protein P300 (EP300), which has histone acetyltransferase (HAT) activity.</td>
</tr>
<tr>
<td>p38</td>
<td>also known as Mitogen-Activated Protein Kinase 14 (MAPK14)</td>
</tr>
<tr>
<td>P53</td>
<td>also known as TP53 (tumour protein p53)</td>
</tr>
<tr>
<td>Pax3</td>
<td>paired box 3 gene</td>
</tr>
<tr>
<td>Pax6</td>
<td>paired box 6 gene; in zebrafish there are two genes pax6a and pax6b; Pax6 gene is in mouse</td>
</tr>
<tr>
<td>pb</td>
<td>proboscipedia gene in fly; it is evolutionary close to HOX in 2nd paralog group</td>
</tr>
<tr>
<td>PBC</td>
<td>Exd/Pbx/Ceh-20 protein family</td>
</tr>
<tr>
<td>PBC-A</td>
<td>PBC-A domain of PBC protein family for interaction with MEINOX proteins</td>
</tr>
<tr>
<td>PBC-B</td>
<td>PBC-B domain of PBC protein family for interaction with MEINOX proteins</td>
</tr>
<tr>
<td>PBX</td>
<td>Pre-B-Cell Leukemia Homeobox; PBX gene family belongs to homeobox gene family termed PBC and encodes PBC domain containing transcription factors.</td>
</tr>
<tr>
<td>PC</td>
<td>pyruvate carboxylase</td>
</tr>
<tr>
<td>PcG</td>
<td>Polycomb group</td>
</tr>
<tr>
<td>PCGF</td>
<td>Polycomb group ring finger (e.g. MEL18, BMI1)</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank (<a href="http://www.pdb.org/pdb/home/home.do">http://www.pdb.org/pdb/home/home.do</a>)</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet endothelial cell adhesion molecule (PECAM-1), which is also known as cluster of differentiation 31 (CD31)</td>
</tr>
<tr>
<td>PEPCK</td>
<td>phosphoenolpyruvate carboxykinase is an enzyme involved in gluconeogenesis</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>proliferator-activated receptor gamma coactivator 1α (also known as PPARGC1A)</td>
</tr>
<tr>
<td>PHC</td>
<td>Polyhomeotic homolog proteins (aka HPHs; e.g. Rae28, HPH1)</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide-3-kinase protein complex</td>
</tr>
<tr>
<td>PIC</td>
<td>PBC Interaction Domain (e.g. YPWM/FDWM/ANW)</td>
</tr>
<tr>
<td>PIN1</td>
<td>peptidylprolyl cis/trans isomerase, NIMA-interacting 1</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A is a protein complex involved in signal transduction pathway downstream of cAMP signaling</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PKNOX (PREP)</td>
<td>PBX/knotted 1 homeobox protein family</td>
</tr>
<tr>
<td>PLA</td>
<td>proximity ligation assay</td>
</tr>
<tr>
<td>Plau</td>
<td>plasminogen activator, urokinase</td>
</tr>
<tr>
<td>pIpb</td>
<td>proteolipid protein 1b gene in zebrafish</td>
</tr>
<tr>
<td>pMN</td>
<td>motor neuron progenitor</td>
</tr>
<tr>
<td>pou3f3b</td>
<td>amacrine marker</td>
</tr>
<tr>
<td>PP1</td>
<td>Ser/Thr protein phosphatase type 1 protein complex</td>
</tr>
<tr>
<td>PP2A</td>
<td>Ser/Thr protein phosphatase type 2A protein complex</td>
</tr>
<tr>
<td>PPRC1</td>
<td>Peroxisome Proliferator-Activated Receptor Gamma, Coactivator-Related 1 (formerly known as PGC-1)</td>
</tr>
<tr>
<td>PR</td>
<td>photoreceptor</td>
</tr>
<tr>
<td>PRC1</td>
<td>Polycomb repressive complex 1</td>
</tr>
<tr>
<td>PRC2</td>
<td>Polycomb repressive complex 2</td>
</tr>
<tr>
<td>ptc2</td>
<td>patched 2 gene encodes a transmembrane receptor of the patched gene family involved in Hedgehog signaling</td>
</tr>
<tr>
<td>Q1/Q2</td>
<td>glutamine-rich domains present in CREB protein family; these domains are important for CREB to interact with TAFII135</td>
</tr>
<tr>
<td>r4</td>
<td>fourth rhombomere</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>Raldh</td>
<td>retinaldehyde dehydrogenase</td>
</tr>
<tr>
<td>RARE</td>
<td>retinoic acid response element</td>
</tr>
<tr>
<td>RARs</td>
<td>retinoic acid receptors</td>
</tr>
<tr>
<td>ras</td>
<td>ras oncogene family encoding small G proteins</td>
</tr>
<tr>
<td>ren</td>
<td>renin gene; it encodes an aspartyl protease involved in blood pressure regulation</td>
</tr>
<tr>
<td>RGC</td>
<td>retinal ganglion cell</td>
</tr>
<tr>
<td>RING</td>
<td>e.g. RING1B, RNF2</td>
</tr>
<tr>
<td>RING1</td>
<td>Ring Finger Protein 1</td>
</tr>
<tr>
<td>rorb</td>
<td>RAR-related orphan receptor B gene</td>
</tr>
<tr>
<td>RPC</td>
<td>retinal progenitor cell</td>
</tr>
<tr>
<td>RPS6KA</td>
<td>Ribosomal protein S6 kinase, 90 kDa, polypeptide (formerly known as RSK)</td>
</tr>
<tr>
<td>rtTA</td>
<td>Mutants of TetR exhibit reverse phenotype, i.e. presence of tetracycline derivatives causes DBD activity, whereas without the drug, there is no DNA binding.</td>
</tr>
<tr>
<td>Runxl</td>
<td>runt-related transcription factor 1 gene</td>
</tr>
<tr>
<td>RXRs</td>
<td>retinoid receptors</td>
</tr>
<tr>
<td>Sca-1</td>
<td>Stem Cell Antigen-1 used as surface marker for HSC; it is a member of Ly-6 antigen</td>
</tr>
<tr>
<td>scl1</td>
<td>also known as T-Cell Acute Lymphocytic Leukemia 1 (tal1) gene</td>
</tr>
<tr>
<td>SCN</td>
<td>suprachiasmatic nucleus</td>
</tr>
<tr>
<td>Scr</td>
<td>Sex combs reduced gene; it is evolutionary close to HOX in 5th paralog group</td>
</tr>
<tr>
<td>shh</td>
<td>sonic hedgehog gene; in zebrafish there are two genes <em>shha</em> and <em>shhb</em> resulted from gene duplication</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA (interference RNA)</td>
</tr>
<tr>
<td>SIK2</td>
<td>salt-inducible kinase 2</td>
</tr>
<tr>
<td>SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>smyhc1l</td>
<td>slow myosin heavy chain 1, like gene in zebrafish</td>
</tr>
<tr>
<td>sox10</td>
<td>SRY (sex determining region Y)-box 10 gene in zebrafish</td>
</tr>
<tr>
<td>splMO</td>
<td>splice morpholino</td>
</tr>
<tr>
<td>SUZ12</td>
<td>SUZ12 Polycomb repressive complex 2 subunit</td>
</tr>
<tr>
<td>T protein</td>
<td>T, Brachyury homolog (mouse) protein</td>
</tr>
<tr>
<td>T2</td>
<td>thoracic segment 2</td>
</tr>
<tr>
<td>TAD</td>
<td>transcription activation domain</td>
</tr>
<tr>
<td>TAFII135</td>
<td>TATA-binding protein associated factor II 135 (also known as TAF4 RNA Polymerase II, TATA Box Binding Protein (TBP)-Associated Factor, 135kDa or TAF4) is a component of transcription factor IID (TFIID) protein complex</td>
</tr>
<tr>
<td>TALE</td>
<td>Three amino acid loop extension; TALE is a conserved three amino acid Pro-Tyr-Pro motif inserted between helices 1 and 2 of the homeodomain, which is a distinguishing characteristics of this gene family.</td>
</tr>
<tr>
<td>TALEN</td>
<td>transcription activator-like effector nuclease</td>
</tr>
<tr>
<td>tbx5</td>
<td>T-box 5 gene; in zebrafish there are two genes tbx5a and tbx5b due to gene duplication</td>
</tr>
<tr>
<td>tfap2d</td>
<td>transcription factor AP-2 delta (activating enhancer binding protein 2 delta) gene</td>
</tr>
<tr>
<td>TFIID</td>
<td>Transcription factor II D protein complex</td>
</tr>
<tr>
<td>TGIF</td>
<td>TGFβ-induced factor homeobox protein</td>
</tr>
<tr>
<td>TILLING</td>
<td>Targeting Induced Local Lesions IN Genomes</td>
</tr>
<tr>
<td>tMO</td>
<td>translation morpholino</td>
</tr>
<tr>
<td>TN</td>
<td>terminal nerve</td>
</tr>
<tr>
<td>trxG</td>
<td>Trithorax group</td>
</tr>
<tr>
<td>TS#</td>
<td>Theiler stage for mouse embryos</td>
</tr>
<tr>
<td>tTA</td>
<td>tetracycline controlled TA (tTA) is a fusion of Tet repressor (TetR) of E. coli (Tn10) and part of the TAD of Herpes simplex protein (VP16). The presence of tetracyclic removes DBD activity of TetR causing the tTA to be unable to bind to DNA, hence no transcription. It is used in Tet-Off system.</td>
</tr>
<tr>
<td>tTA/tetO</td>
<td>tetracycline-controlled transactivator (tTA)/ tetracycline resistance gene operator (tetO) expression system is also known as Tet-Off system, in which gene is repressed in the presence of tetracycline and activated in its absence.</td>
</tr>
<tr>
<td>Ubx</td>
<td>Ultrabithorax in fly; it is evolutionary close to HOX in 7th paralog group</td>
</tr>
<tr>
<td>UEF3</td>
<td>Urokinase enhancer factor 3</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>var</td>
<td>variant</td>
</tr>
<tr>
<td>vax2</td>
<td>ventral anterior homeobox 2 gene in zebrafish</td>
</tr>
<tr>
<td>WNT</td>
<td>Wingless-Type MMTV Integration Site Family</td>
</tr>
<tr>
<td>WRPW</td>
<td>Trp-Arg-Pro-Trp motif; this motif is found at the C-terminal end</td>
</tr>
</tbody>
</table>
of DBD of bHLH repressor (e.g. HES) and it is important for recruitment of corepressors such as Groucho (D. melanogaster)/TLE

| YPWM (or FDWM) | Tyr-Pro-Trp-Met (or Phe-Asp-Trp-Met) motif situated at the N-terminal arm of homeodomain of HOX protein (in paralog groups 1 to 8) which is important for interaction with PBC HD protein; this motif is very similar to ANW found in HOX in paralog groups 9 and 10 |
| zen | zerknullt |
| ZFIN | The Zebrafish Information Network (ZFIN) |
| ZFN | zinc finger nucleases |
| * | stats symbol for p < 0.05 |
| ** | stats symbol for p < 0.01 |
| *** | stats symbol for p < 0.001 |
| **** | stats symbol for p < 0.0001 |
This page intentionally left blank
ABSTRACT

The function of homeobox (HOX) proteins depends on formation of higher order complexes with members of the three-amino-acid-loop-extension (TALE) family of cofactors such as Myeloid Ecotropic Insertion Site (MEIS) and Pre-B-Cell Leukemia Homeobox (PBX). The importance of such complexes is to impart selectivity and specificity to HOX proteins in homing in to their target genes and to confer repressor or activator activity depending on cellular and environmental cues. Furthermore, the function of HOX complexes is regulated by PKA signalling in a manner dependent on interaction with the cAMP responsive element binding (CREB) transcription factor and its co-activators CREB Regulated Transcription Coactivator (CRTC) and CREB Binding Protein (CBP). The biological significance of such interactions has been demonstrated in the context of leukemogenesis; however, much remains to be elucidated in term of embryonic development. Zebrafish is a vertebrate model system which has been extensively used by developmental biologists due to its embryological and genetic tractability. The availability of knockdown technology mediated by antisense morpholino oligonucleotides has been instrumental in the study of gene function during zebrafish embryonic development. Our lab is interested in the study of the cooperative action of HOX-MEIS-PBX complexes with CREB-CRTC during embryonic development. We aim to achieve this by elucidating the genetic interactions of the complex members, particularly MEIS1 and CRTC1. The N-terminal region of CRTC1 has been shown to interact with C-terminal transactivation domain of MEIS1, thus bridging HOX and CREB biological functions. Several labs have characterized the role of meis1 during zebrafish development. However, crtc1 function has not been explored in zebrafish. The aim of this thesis is to characterize the biological role of crtc1 during zebrafish development by a reverse genetics approach using morpholino technology. I have characterized the spatial and temporal expression of crtc1 and crtc1b during zebrafish development, and identified unique splicing of maternal and zygotic specific crtc1b transcripts. Also, I have demonstrated the co-activator function of zebrafish Crtc1 and Crtc1b by using CRE luciferase reporter assay in cell culture. Morpholino knockdown of crtc1 or crtc1b suggested partial functional redundancy of the crtc1 members. Knockdown of crtc1 function provoked a phenotype that overlaps with that of meis1 or creb morphants, such as reduction of the eye and brain. In addition, crtc1 morphants exhibited smaller body size and abnormal body shape (e.g.
“curly-tail-down” phenotype). ISH results demonstrated reduction in cells expressing neural and retinal progenitor/differentiation markers in morphants. Other biological processes were shown to be affected by crtc1 knockdown, namely perturbed notochord maturation (as assessed by persistent expression of shha and ihhb), early dorsal-ventral patterning (as indicated by ventral expansion of gsc expression), and convergence movement (as demonstrated by reduced myod-expressing cell migration toward the dorsal midline). These results provide a platform for future work to elucidate the collaborative role of meis1 and creb-crtc1 during zebrafish embryonic development.
CHAPTER 1: INTRODUCTION

Homeobox (HOX) proteins form complexes with Three-amino-acid-loop-extension (TALE)-containing cofactors, e.g. Meis homeobox (MEIS) and Pre-B-Cell Leukemia Homeobox (PBX), i.e. HOX-PBX-MEIS. Previously, we have demonstrated that the protein complex activity – which plays a role in embryonic development, adult maintenance of hematopoiesis, and pathological development of cancer – is dependent on protein kinase A (PKA) signaling. For example, the HOX-PBX heterodimer can act as either a repressor or activator by differential interaction with co-regulators (e.g. co-repressors or co-activators). The decision has been shown to rest on cAMP-PKA signalling and recruitment of MEIS protein to the HOX-PBX complex (Huang et al., 2005; Saleh et al., 2000b). We have also shown that this activity is also dependent on PKA-activated CREB protein and the concomitant recruitment of its coactivators such as CREB binding protein (CBP) and CREB-regulated transcription coactivator (CRTC), to the HOX-PBX-MEIS ternary complex (Goh et al., 2009; Goh, 2007; Huang et al., 2005; Saleh et al., 2000b). This observation was later corroborated by Wang et al. (2010) in leukemic cells. As an extension to the previous studies, the goal of this research programme presented in this thesis was to study the collaborative interaction of Hox cofactor Meis1 and Creb coactivator Crtc1 during zebrafish development (see section 1.8). In this section, I will provide an overview of Hox genes and their cofactors. Subsequently, I will introduce the Creb family and its coregulators Chp and Crtc. Finally, I will discuss the advantages of using zebrafish as a model organism and the usefulness of morpholino-based knockdown technology to study questions in developmental biology.
1.1.  **Hox genes**

*Hox* genes play a crucial role in the patterning of the embryonic axes during development. They establish a blueprint for positional identity important for subsequent morphogenesis and organogenesis. They were first identified and characterized in fruit fly (*Drosophila melanogaster*) by Edward B. Lewis in 1978. Subsequent molecular analysis revealed that *D. melanogaster* has eight *Hox* genes organized into two clusters, namely Antennapedia complex (ANT-C) and bithorax complex (BX-C), situated in the third chromosome. ANT-C comprises *labial* (*lab*), *proboscipedia* (*pb*), *Deformed* (*Dfd*), *Sex combs reduced* (*Scr*), and *Antennapedia* (*Antp*) genes which are separated by 7.5 Mb intervening sequence from BX-C comprising *Ultrabithorax* (*Ubx*), *abdominal A* (*abd-A*) and *Abdominal B* (*Abd-B*) genes (Fig. 1.1A).

1.1.1.  **Clustered Hox genes**

Clustered *Hox* genes in invertebrates are believed to have originated from hypothetical *Hox* ancestor genes organized in a cluster formed by tandem duplications (Fig. 1.1A; Ferrier, 2007). Cluster organization of *Hox* genes is retained in proto-vertebrate amphioxus and mammals, whereas in *D. melanogaster*, this cluster was split into two sub-clusters. In mammals, there are four *Hox* clusters located in different chromosomes as a result of two rounds of genome wide duplications (reviewed in Ferrier, 2007; Prince and Pickett, 2002). There are a total of 39 *Hox* genes distributed over these clusters, which can be sub-grouped into 13 paralog groups based on molecular phylogeny (Fig. 1.1; Prince and Pickett, 2002; Sharkey et al., 1997). In teleosts, there was an additional whole genome duplication event generating eight *Hox* clusters, but in zebrafish one cluster was subsequently lost during evolution, leaving only seven *hox* clusters (Amores et al., 1998; Meyer and Schartl, 1999). Similar to tetrapods, none of the zebrafish *hox* clusters retains a complete set of thirteen paralog members (Fig. 1.1 B-C). A general guideline for systematic nomenclature of *Hox* genes based on chromosomal location of Hox clusters and paralog group assignments was proposed in 1992 (Scott, 1992, 1993).
Figure 1.1  Hox cluster duplication and gene loss through evolutionary time.

(A) Hypothetical ancestor Hox genes comprises anterior (paralog group 1-5), central (paralog group 6-7) and posterior (paralog group 9-14) Hox genes. In D. melanogaster, members of paralog group 3 are believed to have evolved into non-Hox function (e.g. zen1, zen2 and bcd), whereas amphioxus retained this function (Ferrier, 2010). In addition, amphioxus has additional posterior Hox genes (paralog group 9-14) resulting from multiple tandem duplications. (B) In mouse, two whole Hox cluster duplication events produced four Hox clusters, whereas (C) zebrafish, a teleost has seven hox clusters due to additional round of genome duplication. Loss of duplicated genes resulted in no cluster having a complete set of Hox genes. Open arrow shows the direction of transcription and orientation of Hox genes in the cluster (figure adapted from Prince and Pickett, 2002).
1.1.2. *Hox* colinearity

Clustering of *Hox* genes is accompanied by characteristic colinear expression of *Hox* genes. Colinearity is a term used to describe the ordered activation of each *Hox* gene in a manner directly correlated to the linear order of these genes along the *Hox* cluster. In vertebrates, the term is also used to describe the ordered activation of *Hox* genes relative to both the embryonic anterior-posterior axis and the temporal order of gene activation, and together is known as spatial and temporal colinearity. For example, *Hox* genes located 3’ in the cluster are expressed earlier and in more anterior positions (e.g. in rhombomeres) than subsequent genes located more 5’ in the cluster, with the 5’ most *Hox* genes being expressed later and in more posterior positions (Durston et al., 2011). In *D. melanogaster*, spatial colinearity of *Hox* expression is observed whereas temporal colinearity is not (Ferrier, 2007). There are a few exceptions to the colinearity rule (reviewed in Nolte and Krumlauf, 2007).

1.1.3. HOX code

In vertebrates, *Hox* genes are expressed in broad overlapping domains along embryonic axes producing nested expression of HOX proteins. The combinatorial expression of different *Hox* genes (or “HOX code”) has been suggested to play a role in the patterning of different body segments along these axes. For example, during the development of the murine vertebral column, cervical pre-vertebra C1 identity is dependent on the HOX code comprising paralogs 1 to 3 and *Hoxd4* whereas C2 identity is dependent on paralogs 1 to 3, *Hoxa4, Hoxb4*, and *Hoxd4* (Fig. 1.2; Pang and Thompson, 2011). Disruption of these codes has been shown to produce homeotic transformation of the respective vertebra. For instance, in *Hoxd4* hetero- or homozygous mutant mice, disruption of *Hoxd4* gene function in C1 and C2 caused abnormal fusion of the basioccipital bone and homeotic transformation of C2 to C1 identity (as assessed by vertebral morphology) in newborn mice, respectively (Horan et al., 1995a). Similarly anterior transformations were observed in single *Hoxa4<sup>−/−</sup>* (C3 to C2 transformation) and *Hoxb4<sup>−/−</sup>* (anterior transformation of C1 similar to *Hoxd4* mutation) mutants (Horan et al., 1994; Ramirez-Solis et al., 1993). Even more strikingly, *Hoxa4, Hoxb4, and Hoxd4* compound mutant mice exhibited extensive
anterior transformation covering several pre-vertebrae (C2 to C4) as compared to partial transformation in single mutants, which suggests cooperative and complementing roles of the fourth paralogs in these segments (Horan et al., 1995b). This functional redundancy has been noted in other paralogous genes as well (reviewed in Maconochie et al., 1996).

Another unique feature about the HOX code is that posterior HOX function can override anterior HOX function (“posterior prevalence”) when co-expressed, and this usually leads to posterior transformation of body segment (Duboule and Morata, 1994; Favier and Dolle, 1997; Gonzalez-Reyes and Morata, 1990). The general view is that loss-of-function Hox mutations normally cause anterior transformation of posterior segments while the corresponding gain-of-function mutations lead to posterior transformations of anterior structures. However, such “global posterior prevalence” is not a hard and fast rule and seems to be exclusive to vertebrates. Several explanations have been given (cf. anterior prevalence; reviewed in Durston, 2012).

1.1.4. HOX protein domains

1.1.4.1. HOX Homeodomain

Hox genes are relatively small, usually comprising just two coding exons, with the second exon containing the homeobox. The homeobox is a highly conserved 180 base pair (bp) DNA sequence which encodes a 60 amino acid homeodomain (HD) important for DNA binding activity of all HOX proteins (McGinnis et al., 1984; Muller et al., 1988; Scott and Weiner, 1984). This homeobox is not exclusive to the clustered Hox genes and is found in the genes encoding other transcription factor families. Notably, this includes members of the TALE family to be discussed below (Scott et al., 1989). The homeobox sequence was first found in D. melanogaster Hox genes and has been instrumental in uncovering many other genes harbouring the homeobox sequence.
Figure 1.2 Spatial colinear expression of murine *Hox* genes along the embryonic vertebral column

Anterior boundary of *Hox* genes expression domains is shown (highlighted in red). Each prevertebral segment has a characteristic combination of *Hox* genes expression (*Hox* code) that specifies its segment identity. For example, HOX code for C1 comprises *Hox* paralogs 1 to 3 and *Hoxd4* (highlighted cyan) and C2 is similar to C1 but with additional expression of *Hoxa4* and *Hoxb4* (highlighted yellow). Genes located more 3’ (anterior genes) are expressed more anteriorly as compared to subsequent genes 5’ of the *Hox* cluster, which clearly showed the spatial colinearity of *Hox* expression (embryonic age not mentioned; Favier and Dolle, 1997; Pang and Thompson, 2011). In comparison to E12.5 mice, the anterior border of *Hoxb4* and *Hoxd4* is at C1/2, followed by *Hoxa4* at C2/3 and *Hoxc4* at C3/4 (Horan et al., 1995b). Figure is adapted from Favier and Dolle (1997) and Pang and Thompson (2011).
Based on X-ray crystallography and nuclear magnetic resonance (NMR) analyses of HD-DNA complex, the HOX HD structure is composed of a flexible N-terminal arm (also known as “N-terminal tail” of HD) which is followed by three alpha helices. Helix 2 and helix 3 (the latter also known as the DNA recognition helix) are connected by a turn, thus forming the helix-turn-helix structure as also found in some bacterial repressors (Fig. 1.3A-B). The DNA binding activity of the HD is accomplished by the N-terminal arm and helix 3 which form hydrogen-bond contacts in the minor and major groove of DNA, respectively (Kissinger et al., 1990; Qian et al., 1989). The N-terminal arm is usually disordered in the absence of DNA, but assumes a regular structure upon DNA binding. The flexible N-terminal arm is also believed to assist the HD to search the DNA for binding sites and subsequently to stabilize HD-DNA interaction (facilitated diffusion and "fly casting" models reviewed in Toth-Petroczy et al., 2009; Vuzman et al., 2010).

1.1.4.2. YPWM/ANW motif

HOX proteins from paralog groups 1 to 8 share a conserved YPWM (or FDWM in HOX paralog group 1) motif located N-terminal to the HD. In HOX proteins from paralog groups 9 and 10, this motif is replaced by a similar tryptophan-containing sequence, ANW. These motifs (recently grouped as the PBC Interaction Domain or PID) are important for cooperative DNA binding activity of HOX proteins from paralog groups 1 to 10 (but not paralog groups 11 to 13 which lack these motifs) with the PBC family of HD-proteins (see section 1.1.6.) (Shen et al., 1997a). The YPWM/ANW motif of HOX proteins has been shown to interact with the hydrophobic pocket located in the HD of TALE (three amino acid loop extension) class transcription factor PBX (or EXD in flies) (Fig. 1.3B; see section 1.2.1.). The HOX YPWM motif folds into a preformed structure for PBX interaction and mutating this motif abolishes HOX-PBX cooperative binding to DNA (without affecting monomeric DNA binding activity) (Bourdon et al., 2005; Chang et al., 1996; Knoepfler and Kamps, 1995; Lu and Kamps, 1996; Neuteboom et al., 1995; Phelan and Featherstone, 1997; Phelan et al., 1995; Slupsky et al., 2001). Furthermore, mice with a targeted mutation in the YPWM motif of Hoxa1 show a mutant phenotype reminiscent of Hoxa1 knockout mice, underscoring the importance of this motif for Hoxa1 function.
(Remacle et al., 2004). An additional motif important for interaction of the fly HOX protein UBX with EXD has also been reported (reviewed in Mann et al., 2009).

Figure 1.3 Primary, secondary and tertiary structure of the HD

(A) The primary and secondary structures of the HD of UBX in comparison to the murine HOX proteins (HOXA1, HOXD4, HOXA6 and HOXD9). The unstructured N-terminal arm and three alpha helices are shown as blue and orange lines, respectively. (B) Crystal structure of HOXB1 and HOX cofactor PBX in complex with DNA (derived from PDB ID: 1B72). The N-terminal arm and helix 3 contact the minor and major grooves of DNA. Upstream of the N-terminal arm is the YPWM motif (highlighted green) that contacts the hydrophobic pocket of the PBX HD. The fourth alpha helix of PBX formed upon DNA binding is labelled (adapted from Passner et al., 1999; Piper et al., 1999).
1.1.5. HOX monomeric DNA binding specificity

The HOX HD binds to DNA sequences containing the core recognition site 5’-TAAT-3’ (or “core”). The N-terminal arm of HD binds to the first two bases of 5’-TAAT-3’ located in the minor groove of DNA. Several basic residues in the N-terminal arm, such as at position 2, 3 and 5 of the HD, are required for this interaction (Ades and Sauer, 1995; Featherstone, 2003; Kissinger et al., 1990). The absence of basic residues at position 2 and 3 of the HD as that found in Hox paralog group 1, reduces monomer binding affinity and therefore differentiates the binding activity of paralog group 1 from other HOX proteins (Featherstone, 2003; Phelan and Featherstone, 1997). In the major groove of DNA, Helix 3 form extensive hydrogen-bond contacts with the DNA bases and with the phosphate backbone. Several residues important for this process are highly conserved in Hox proteins, e.g. Ile-47, Gln-50, Asn-51, and Met-54 which make specific contacts with DNA sequences containing the core (Featherstone, 2003; Kissinger et al., 1990; Mann, 1995). For example, Asn-51 contacts DNA at the third base of 5’-TAAT-3’, whereas Gln-50 makes contact with two bases downstream of 5’-TAAT-3’, e.g. 5’-TAATNN-3’ (Featherstone, 2003). These highly conserved DNA binding residues in helix 3 are responsible for constraining the DNA binding site selectivity of HOX proteins. Although there are subtle differential binding preferences and affinities afforded by variations at other residues in the HDs of the various paralogs, the effect is often minimal (Pellerin et al., 1994). Furthermore, the HOX sequence recognition site 5’-TAAT(g/t)(g/a)-3’ occurs at a high frequency of once every 500 base pair in the genome (Galant et al., 2002). It is therefore unlikely that HOX proteins alone are sufficient to discriminate their target genes, although there are exceptions where HOX protein functions as monomers (Featherstone, 2003; Galant et al., 2002).

1.1.6. Cooperative DNA binding with HOX cofactors

Although HOX proteins can function as monomers, they can also interact with other DNA binding proteins to form complexes that bind to DNA. As mentioned above, one such protein is encoded by PBX, or extradenticle (EXD) in flies. Another is MEIS, or homothorax (HTH) in flies. Both PBX/EXD and MEIS/HTH are members of the
ataypical HD family called TALE, which stands for “three-amino-acid-loop-extension”. TALE family members are characterized by a divergent HD having an extra conserved three amino acids (Pro-Tyr-Pro) inserted between helices 1 and 2 (Bürghlin, 1997).

There are several advantages for HOX proteins to form higher order protein complexes with their cofactors. First, considering that 5’-TAAT-3’ sequence bound by monomeric HOX occurs rather frequently in the genome, the formation of higher-order DNA binding complexes allows for longer DNA binding sites, and hence increasing DNA binding affinity as well as specificity (rather than relying on chance). For example, HOX-PBX heterodimers have been shown to bind to a bipartite sequence 5’-ATGATTTNATNN-3’ (which would occur by chance once every 8200 base pairs as estimated by Galant et al., 2002), in which PBX binds to the 5’-TGAT-3’ half-site and HOX protein binds to the second half-site 5’-TNATNN-3’ (Chang et al., 1996; Laurent et al., 2008). Also, HOX-PBX interactions have been shown to have the ability to change HOX DNA-binding selectivity, and hence distinguish DNA-binding specificities of different HOX proteins (Featherstone, 2003; Slattery et al., 2011). This is probably accomplished by causing conformation changes to the N-terminal arm of HOX HD when its upstream YPWM/ANW motif interacts with PBX (Slattery et al., 2011). For example, while monomeric HOXD4 has a preference for a 5’-TAAT-3’ core, this is altered to encompass 5’-TDAT-3’ (D = A/G/T) in a HOXD4-PBX heterodimer.

PBX has been shown to recruit co-repressors to HOX-PBX heterodimer, suggesting that HOX activity can be modulated by PBX (Asahara et al., 1999; Saleh et al., 2000b). The recruitment of co-repressors to the N-terminus of PBX1 is thus antagonistic to activator function supplied by the HOX partner through recruitment of coactivators (e.g. CBP). HOX-PBX function is switched from repressor to activator by PKA signaling (Saleh et al., 2000b). I will return to the importance of PKA to the function of HOX complexes below.

Differential interaction of HOX paralogs with PBC proteins has been observed, in which HOX proteins belonging to paralog groups 1 to 10 form DNA-binding complexes with PBX/EXD proteins (which belong to PBC class proteins as will be discussed in section 1.2.1) but not HOX proteins from paralog groups 11 to 13, which
do not have a distinguishable YPWM/ANW motif. MEIS family HD proteins, like PBX, are members of the TALE family and HOX partners. MEIS proteins have been shown to cooperatively bind to DNA targets with HOX paralog groups 9 to 13 (Chang et al., 1996; Shen et al., 1997a; Shen et al., 1997b). This interaction requires the C-terminus (inclusive of HD) of MEIS proteins and N-terminal (involving multiple peptide domains) of HOX proteins (Shen et al., 1997b; Williams et al., 2005). Furthermore, unlike HOX-PBX, MEIS1A-HOXA9 complexes can form in the absence of DNA (Shen et al., 1997b). The differences in the HOX cofactor interaction among HOX paralog groups suggest a probable means to distinguish the anterior from posterior HOX functions. Moreover, paralog groups 9 and 10 which are able to bind to both MEIS and PBX proteins would be expected to have distinct functions relative to other paralog groups. However, one report provides evidence that a broader array of HOX-PBX complexes is capable of recruiting MEIS as both a DNA-binding or non-DNA binding partner (Shanmugam et al., 1999). Interestingly, cooperative DNA binding of HOX-PBX-MEIS heterotrimers would increase their specificity even further (e.g. HOX-PBX-MEIS binding sites would be expected to occur once every 420,000 base pair as estimated by Galant et al., 2002; Laurent et al., 2008).

1.1.7. Regulation of Hox expression

Regulation of Hox genes expression has come to be regarded as one of the crucial factors influencing specificity of HOX function (Featherstone, 2003). Thus, substantial effort has been made to understand the regulatory mechanisms (or influences) that modulate Hox gene expression. The ontogenesis of Hox expression can be divided into several stages such as initiation, establishment and maintenance (Deschamps et al., 1999). The initiation stage is defined as the time when Hox genes are first induced, which is then followed by an interval period (establishment) needed to stabilize Hox genes expression (maintenance) with definitive expression boundaries (Deschamps et al., 1999).

In early vertebrate development, Hox clusters are maintained in a globally silent state, and then become progressively activated in a temporal sequence from a 3’ to 5’ direction (temporal colinearity). Early Hox genes are activated during
gastrulation in the non-organizer mesoderm (also known as “Hox induction field”) (Deschamps et al., 1999; Gaunt, 2000; Wacker et al., 2004a), induced by the coordinated action of several signaling pathways and upstream regulators. In African clawed frog (Xenopus laevis), Bmp4 and T protein (formerly Brachyury) are sufficient to induce Hox gene expression in the non-organizer mesoderm, in a temporally colinear fashion (Wacker et al., 2004a). Temporally colinear Hox expression in this tissue is important for subsequent spatial patterning of Hox genes along the anterior-posterior axis. In the “time space translation” model, the anterior-posterior patterning of Hox gene expression in mesodermal tissue is believed to be formed by successive involution of temporally colinear HOX-code-expressing mesodermal cells during axis elongation. In other word, each involuting mesodermal cell that leaves the Hox induction field at a specific time will retain its unique HOX code, as it is unable to further activate new Hox genes while away from the inductive field. The last involuting cells with the longest exposure to the inductive signal will express most of the posterior Hox genes (Durston et al., 2012; Wacker et al., 2004b). The resulting anterior-posterior HOX pattern in paraxial mesoderm is then copied to the overlaying neuroectoderm by vertical signaling, and refined by the planar signals emanating from the organizer (Durston et al., 2012; Wacker et al., 2004b). In addition to Bmp4 and T proteins in X. laevis Hox induction, several other upstream regulators have been implicated in the initiation of Hox genes expression (see below).

Retinoic acid (RA) signaling is important for providing positional information for embryos during early development. Disruption of RA signalling has been known to cause anterior-posterior transformation of the X. laevis body axis in both mesodermal and neuroectodermal tissues (Durston et al., 1989; Ruiz i Altaba and Jessell, 1991a; Ruiz i Altaba and Jessell, 1991b; Sive et al., 1990). For example, too much RA causes posteriorization whereas RA deficiency produces anteriorized embryo. RA signalling is mediated by its binding to retinoic acid receptors (RARs) and retinoid receptors (RXRs), which form heterodimeric transcription factors. In vertebrates, there are three RARs (alpha, beta, and gamma) which binds to both RA derivatives (i.e. all trans-RA and 9-cis RA) and three RXRs (alpha, beta, and gamma) which only bind 9-cis RA. RAR-RXR heterodimers modulate transcriptional activities of their target genes by binding to regulatory regions in DNA bearing a consensus sequence named the retinoic acid response element (RARE). RA signaling has been shown to be essential
for the temporal colinear expression of *Hox* genes in human embryonal carcinoma cells, in which RA treatment causes sequential activation of anterior *Hox* genes followed by posterior genes (Simeone et al., 1990). In mouse, RA has been demonstrated to be crucial for the proper spatial expression of *Hox* genes. In *Raldh2* homozygous null mice which cannot synthesize RA, anterior *Hox* gene expression in the hindbrain is caudalized whereas ectopic RA (by maternal) administration causes anteriorized *Hox* genes expression during hindbrain development (Conlon and Rossant, 1992; Niederreither et al., 2000). Several *Hox* genes have been found to carry RAREs in their regulatory regions, some of which can exert long range cis-transcriptional control over other *Hox* genes (reviewed by Featherstone, 2003). The functional significance of RAREs in *Hox* regulatory elements have been shown to be involved in maintaining proper temporal and spatial expression of *Hox* genes (Dupe et al., 1997; Gould et al., 1998; Huang et al., 1998; Oosterveen et al., 2003). Other signaling pathways such as fibroblast growth factor (FGF) and canonical wingless-type MMTV integration site family (WNT) have been shown to affect *Hox* gene expression and patterning of the embryonic axis (Aulehla and Pourquie, 2010; Dollar and Sokol, 2007; Greco et al., 1996; Partanen et al., 1998).

Several upstream regulators important for *Hox* genes expression have been identified, namely Early Growth Response 2 (EGF2; formerly known as KROX20; Sham et al., 1993), v-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog B (MAFB; formerly known as KREISLER or KRML; Manzanares et al., 1999), Kruppel-like factors (KLF; Doerksen et al., 1996; Maconochie et al., 1999), and Caudal type homeobox (CDX) proteins (reviewed in Aulehla and Pourquie, 2010; Deschamps and van Nes, 2005), among others. EGF2, MAFB, and CDX proteins act as positive regulators, whereas KLF can negatively or positively regulate *Hox* genes expression, depending on the cellular context. Interestingly, CDX homeodomain proteins (e.g. murine CDX1, CDX2 and CDX4), which have been shown to directly activate *Hox* genes, are direct targets of RA and FGF signaling (reviewed in Aulehla and Pourquie, 2010). Loss of CDX protein function in mouse embryos has been shown to cause anterior transformation of the axial skeleton, with a concomitant posterior shift of *Hox* gene expression (Subramanian et al., 1995; van den Akker et al., 2002).
Hox gene expression can also be regulated via autoregulation and cross-regulation. In autoregulatory mode, Hox gene expression is regulated by their respective products. For example, Hoxb1 and Hoxal expression is initially induced by RA acting on an RARE-containing element 3’ of these genes in mouse hindbrain. The early expression of these HOX proteins in turn reinforces the expression of Hoxb1 by auto- or cross-regulatory interaction at an autoregulatory element (ARE) that contains HOX-PBX binding sites located 5’ of Hoxb1, (Popperl et al., 1995). In addition, HOXB1 is also required for cross activation of other Hox genes (e.g. Hoxa2 and Hoxb2) in r4 via enhancer regions consisting of HOX-PBX (and MEIS/PKNOX) binding sites (Ferretti et al., 2000; Jacobs et al., 1999; Maconochie et al., 1997).

Stable Hox gene expression is maintained at the chromatin level via epigenetic regulation. This is accomplished by marking the transcriptional activity in the Hox cluster by either repressive- or active- histone modifications (or “histone codes”), which can then be inherited over several rounds of the cell cycle. The histone methyltransferases Polycomb group (PcG) and trithorax group (trxG) have been shown to be important in this process. PcG are multimeric repressive chromatin modifiers that can be divided into two classes, Polycomb repressive complex 1 (PRC1) and PRC2. The PRC1 complex is composed of CBX proteins, the PCGF family proteins (PCGF1–PCGF6), the RING1 family proteins (RING1a and RING1b) and the Polyhomeotic homolog proteins (PHC1 to PHC3; formerly known as HPHs), whereas the PRC2 complex is made up of EZH2 (or its close homolog EZH1), EED and SUZ12 (reviewed in Di Croce and Helin, 2013). A model for PRC1 and PRC2 actions have been proposed whereby PRC2 is first recruited to gene regulatory region where it causes histones deacetylation (by recruitment of HDAC) followed by conversion of histones to repressive marks by histone methylation. This is then succeeded by recruitment of PRC1 to this site to remodel the chromatin into a closed chromatin state (Kobrossy and Featherstone, 2007; Simon and Tamkun, 2002). Mutations of several components of PRC1 such as CBX (M33), PCGF members (e.g. MEL18 and BMI1), PHC members (Rae28/HPH1), and RING members (e.g. RING1B/RNF2) have been shown to perturb Hox genes expression patterns (Kobrossy and Featherstone, 2007). To add to the complex nature of Hox genes regulation, long non-coding RNAs have also been found to regulate Hox expression by means of recruiting Polycomb group repressive complexes (Dasen, 2013; Delpretti et al., 2013; Li et al., 2013; Rinn et al.,
On top of that, Hox genes are regulated from a distance by remote and global cis-regulatory regions (reviewed in Spitz, 2010) and post-transcriptionally by microRNAs (reviewed in Yekta et al., 2008).

### 1.1.8. HOX function

Hox genes play an important role during embryonic development by specifying positional identities along the anterior posterior axis. Perturbation of HOX function (or HOX code) has been shown to cause homeotic transformation of body parts, e.g. the classical loss of Ubx function in *D. melanogaster* that causes anterior transformation of thoracic segment 3 (T3) to T2, in which the haltere in T3 is transformed to wing resembling T2 (Lewis, 1978). Experimental manipulations (e.g. knock out technology, RA induction, etc.) on Hox genes in vertebrates yield similar observations, suggesting that Hox genes function in patterning animal body is evolutionarily conserved. However, not all the phenotypes resulting from HOX loss of function can be interpreted as homeotic transformation, suggesting that Hox genes also play additional roles in organogenesis.

Hox genes have been shown to be important for the development of the vertebrae (see section 1.1.3.) and the central and peripheral nervous systems (reviewed in Sanes et al., 2011). In mice, Hox genes in paralog groups 1 to 4 are expressed in the hindbrain with different anterior borders and overlapping expression patterns. This produces a specific HOX code for each rhombomere (Fig 1.4). Deletion of the Hoxa1 gene in mice causes rhombomere 5 and 6 to merge and reduces the r4 territory. There is a loss in facial motor neurons originating from these rhombomeres as well. In other mutant mice with loss of Hoxb1, r4 identity is affected, as is cranial nerve development. The most striking defect can be seen in Hoxa1 and Hoxb1 double mutant mice, in which there is even greater loss of facial motoneurons than either single knockout mice (reviewed in Goh, 2007; Sanes et al., 2011). Hox gene function in patterning and development of the vertebrae has been demonstrated in mice bearing compound mutations of Hox paralog group 4 genes. Mice lacking Hoxa4, Hoxb4, and Hoxc4 show an extended homeotic transformation of prevertebrae C2 through C5 to C1 identity (see section 1.1.3; Horan et al., 1995b).
Hox genes are also involved in vertebrate limb development, in which Hox genes from A and D clusters are believed to play important role, whereas homozygous deletions of either the Hox B cluster (perinatal lethal, with axial patterning defects, but no mention of limb defect) or Hox C cluster (perinatal lethal with minor transformations and no limb defect) have been shown to be dispensable for limb development in the mouse (Medina-Martinez et al., 2000; Suemori and Noguchi, 2000). Hox members of the same paralog groups in Hox A and D clusters show similar expression domains in the limb bud. They are expressed in a temporal colinear fashion, in which anterior Hox genes are expressed first followed by more posterior Hox genes. This produces a nested expression of Hox genes, in which the most anterior Hox genes are expressed in a more anterior-proximal region of the limb bud, followed by series of nested expressions of subsequent posterior Hox genes expressed at more posterior-distal region (in a pattern analogous to “Russian dolls”) (based on chick and mouse data; Haack and Gruss, 1993; Nelson et al., 1996). At later stages of limb development, a second pattern emerges in which posterior Hox genes are expressed more distally in the limb bud as compared to anterior Hox genes. Unlike the classical homeotic transformation of the trunk, disruption of Hox genes function in the limb bud normally involves loss (or hypoplasia) of skeletal elements. For example, disruption of Hox paralog group 11 (e.g. Hoxa11−/−;Hoxd11−/−) function causes shortening of zeugopods (e.g. ulna and radius) in mice (Davis et al., 1995), whereas combined removal of Hox paralog group 13 (e.g. e.g. Hoxa13−/−;Hoxd13−/−) resulted in loss of autopods in mice (Fromental-Ramain et al., 1996a). Similarly, perturbation of more anterior Hox genes (e.g. paralog groups 9 or 10) causes hypoplasia to more proximal structures (Fromental-Ramain et al., 1996b; Graham, 1994; Zakany and Duboule, 2007).

Hox genes have also been shown to be involved in hematopoiesis. For example, murine Hox genes from cluster A (e.g. Hoxa4, Hoxa5, Hoxa9, and Hoxa10) and B (Hoxb2, Hoxb4, and Hoxb9) are expressed abundantly in hematopoietic stem cells (HSCs; e.g. Sca-1/Ly6a+ lineage markers [lin]+ subpopulation) from bone marrow and fetal liver, whereas these genes are downregulated in more mature blood cell populations (e.g. Sca-1+ Lin+ and Sca-1− Lin+ subpopulations), consistent with previous results showing that Hox genes expression is abundant in blood stem cells and reduced in differentiated cells (Pineault et al., 2002; Sauvageau et al., 1994). In comparison, Hox cofactors such as Meis1, Pbx1, and Pbx2 are detected at higher levels
in Sca-1+ Lin- fraction in both bone marrow and fetal liver (similar to Hox genes), whereas in more mature cell populations (e.g. Sca-1+ Lin- and Sca-1- Lin-), Meis1 is markedly reduced while Pbx1 and Pbx2 remain readily detected in these cells (Pineault et al., 2002).

Pbx1 has been implicated in pre-B leukemia as part of the E2A-Pbx fusion gene associated with the chromosomal translocation [t(1;19)(q23;p13)] (see section 1.2.1; Kamps et al., 1990; Nourse et al., 1990). Meis1, on the other hand, was identified based on studies of the myeloid leukemia that frequently develops in BXH-2 mice in which Meis1 expression is usually aberrantly upregulated along with Hoxa7 or Hoxa9 (Nakamura et al., 1996).

Hoxa9 gene is the most highly expressed Hox family member in HSC. Inactivating Hoxa9 function in homozygous null mice caused reduced proliferation and repopulating ability of Hoxa9−/− bone marrow cells. For example, Hoxa9−/− mutant mice failed to recover from a sub-lethal X-ray irradiation and suffered deficiency in all types of blood cells (pancytopenia), as compared to wild-type mice. In addition, Hoxa9−/− bone marrow cells showed 4- to 12-fold reduced repopulation ability in competitive transplantation assays with wild-type cells in lethally irradiated host mice (Lawrence et al., 2005). However, Hoxa9−/− bone marrow cells showed comparable HSC composition and HSC homing to marrow appeared normal, suggesting that Hoxa9−/− HSC has a qualitative defect rather than quantitative. Indeed, Hoxa9−/− KLFS cells (c-kit+ Lin− flk-2− Sca-1+) showed diminished proliferation as assessed by a quantitative clonal assay showing a significant reduction of high-proliferative potential colonies as compared to wild-type (Lawrence et al., 2005).

Enforced expression of Hoxa9 in murine primitive bone marrow cells by retrovirus-mediated gene transfer confers higher repopulation capacity, increased myelopoiesis at the expense of lymphopoiesis (based on clonal analysis of hematopoietic tissues, thymus, or spleen), and a high competitive repopulation unit (as described in Harrison, 1980; Szilvassy et al., 1990) in lethally irradiated host mice (Ly5.1) (Thorsteinsdottir et al., 2002). Co-expression of Hoxa9 and Meis1a in primitive HSC increased the incident of acute myeloid leukemia (AML) in transplanted host mice as early as 49 days post-transplantation, but not in recipients of
bone marrow cells transduced with either Hoxa9, Pbx1b, Meis1a or a combination of Hoxa9 and Pbx1b which survived >170 days post transplantation, although some recipients of cells transduced with Hoxa9 (or Hoxa9 + Pbx1b) developed AML (Kroon et al., 1998). Although Pbx1 did not potentiate Hoxa9 transforming activity, it could still be involved in Hoxa9- and Meis1-induced acute myeloid leukemia, especially considering that endogenous Pbx1 is present. Hence, removal of Pbx1b (or Pbx1) function is necessary to test the importance of Pbx1 in Hoxa9- and Meis1-induced acute leukemia. Furthermore, unlike Pbx1b, co-expression of Hoxa9 and E2A-Pbx1a has been shown to be acutely transforming (Thorsteinsdottir et al., 1999). All in all, HOX genes and their partners play an important role in normal hematopoiesis and dysregulation of their function is detrimental in cases of leukemias (for review, refer to Alharbi et al., 2013; Grier et al., 2005).

Figure 1.4 Hindbrain HOX code

Cartoon showing the anterior borders and overlapping expression patterns of Hox genes from paralog groups 1 to 4 (blue bars) in a mouse hindbrain. Rhombomeres 1 to 8 (r1 to r8) are shown with midbrain situated on the left (adapted from Goh, 2007; Lumsden and Krumlauf, 1996; Sanes et al., 2011).
1.2. HOX cofactors: TALE members

TALE superfamily (or superclass) members are characterized by having a variant HD that contains three extra residues between helix 1 and helix 2. There are five TALE family members identified based on molecular phylogenetic studies, namely MEIS (or MEINOX), PBC, IRO, TGIF and MOHAWK (MKX) (Mukherjee and Burglin, 2007). Of these, MEINOX and PBC are known HOX cofactors. There are also other HOX non-HD cofactors identified, which will not be discussed here (reviewed in Moens and Selleri, 2006).

1.2.1. PBC class protein: PBX

The first clue to PBX as a HOX cofactor came from a study in *D. melanogaster*. For example, in embryos homozygous null for *extradenticle* (*exd*, a *Pbx* ortholog), homeotic transformation of multiple body segments (e.g. from thoracic to abdominal) is observed and resembles loss-of-function of several *Hox* genes. However, the expression of *Hox* genes is not perturbed. Furthermore, when the *Hox* gene *Ubx* is ectopically expressed in *exd* homozygous null background, *Ubx* fails to induce homeotic transformations, suggesting that *exd* and *Hox* function in parallel (Peifer and Wieschaus, 1990). Subsequent experiments show that EXD can modulate HOX (e.g. UBX and ABD-B) DNA-binding specificity and target selection by forming HOX-EXD complexes with DNA (van Dijk and Murre, 1994). Sequence comparison shows that *exd* is the ortholog of human proto-oncogene *PBX1* (Rauskolb et al., 1993). *PBX1* was first identified as a causative factor in human pre-B-cell acute lymphoblastic leukemia due to chromosomal translocation involving chromosome 1 and 19 [t(1;19)(q23;p13)], which results in production of a fusion transcript encoding an E2A-PBX1 chimera (Kamps et al., 1990; Nourse et al., 1990). *PBX2* and *PBX3* were isolated from a human cell line cDNA library based on sequence homology to *PBX1* (Monica et al., 1991). Predicted PBX2 and PBX3 proteins share 92 and 94% identity to PBX1 across 266 amino acids encompassing the HD (Monica et al., 1991). There are several isoforms for PBX1 and PBX3 (but not PBX2), possessing distinctive C-termini due to alternative splicing (Di Rocco et al., 1997; Monica et al., 1991). The C-terminus of PBX1A isoform is able to recruit corepressors whereas PBX1B, which...
lacks a C-terminal subdomain, is unable to interact with these factors (Asahara et al., 1999). Interestingly, the spatio-temporal expression of these transcript variants is differentially regulated. For example, in immunohistochemical analysis, PBX1A isoform is found predominantly in developing neural tissues, whereas the PBX1B isoform is found throughout all tissues (Schnabel et al., 2001). Another member, PBX4, was later isolated in human and mouse which is expressed exclusively in the testis (Wagner et al., 2001). Zebrafish pbx4/lazarus, which was uncovered in forward genetic screen, is orthologous to the mammalian Pbx1 (Popperl et al., 2000; Vlachakis et al., 2000).

There are four mammalian Pbx genes (Pbx1 to Pbx4), five in zebrafish (pbx1a, pbx1b, pbx2, pbx3a, pbx3b, and pbx4), one in D. melanogaster (exd), and one in roundworm Caenorhabditis elegans (Ceh-20) which are grouped into the PBC family based on a conserved PBC domain present at a region N-terminal to the HD (Burglin and Ruvkun, 1992; Laurent et al., 2008). The PBC domain consists of PBC-A and PBC-B sub-domains. Both PBC-A and PBC-B have been shown to be important for protein-protein interaction with PKNOX (formerly known as PREP) and MEIS proteins (Abu-Shaar and Mann, 1998; Berthelsen et al., 1998b; Chang et al., 1997; Knoepfler et al., 1997). Unlike complexes with HOX proteins, PBX interaction with MEIS or PKNOX is DNA-independent (Berthelsen et al., 1998a; Chang et al., 1997; Knoepfler et al., 1997).

PBX function can be regulated by altering its subcellular localization, and this depends on MEIS function, and vice versa. This is based on several observations. First, loss of Meis ortholog HTH function in flies adversely affected EXD nuclear localization and caused cytoplasmic retention of EXD in many embryonic tissues. Secondly, co-expression of EXD and HTH is required for their nuclear localization. Hence, in embryonic segments (e.g. labial and limb primordia) where hth is not expressed, EXD remains cytoplasmic, whereas in embryonic segments (e.g. leg and antennal imaginal discs) where hth is expressed, EXD is localized in the nucleus (Rieckhof et al., 1997). Most importantly, ectopic expression of either hth or Meis1 induces a cytoplasmic-to-nuclear translocation of EXD in insect cells where EXD is normally cytoplasmic (Berthelsen et al., 1999; Kurant et al., 1998; Rieckhof et al., 1997). The nuclear localization of PBX/EXD or MEIS/HTH is dependent on PBX-
MEIS (or EXD-HTH) complex formation. There are two nuclear export signals (NES) located in the conserved PBC-A domain N-terminal to the PBX/EXD HD (Berthelsen et al., 1999; Kilstrup-Nielsen et al., 2003), and two nuclear localization signal (NLS) located within the HD, i.e. within the N-terminal arm and helix 3 (Abu-Shaar et al., 1999; Berthelsen et al., 1999; Saleh et al., 2000a; Stevens and Mann, 2007). The balance between the activity of NES and NLS, mediated by importin (e.g. Karyopherin) and exportin (e.g. CRM1) proteins is important to decide PBX subcellular localization. Formation of the PBX-MEIS complex is believed to mask (or occlude) the NES activity while retaining NLS activity, and hence a net nuclear import of the protein complex (Abu-Shaar et al., 1999; Berthelsen et al., 1999). Another level of control has been shown to rely on the sequestration of PBX/EXD by cytoplasmic protein non-muscle myosin, which can compete with MEIS to retain PBX in the cytoplasm (Huang et al., 2003). Furthermore, PBX1 nuclear export is regulated by protein kinase A (PKA) activity as well. PKA phosphorylation of PBX1 at conserved Ser residues located within PBC-B subdomain prevents PBX1 nuclear export, suggesting that PBX1 nuclear localization can also be regulated independent of MEINOX proteins (Kilstrup-Nielsen et al., 2003).

In adult mouse microarray analysis, *Pbx1* is found expressed abundantly in brain tissues, retina, uterus, ovary, and glandular tissues. *Pbx2* is ubiquitously expressed in other tissues but it is more abundant in blood cells. *Pbx3* is predominantly expressed in the eye region, ovary, retina, pineal gland, and other glandular tissues. *Pbx4* expression is comparable in all tissues, but highest in testes (http://biogps.org). *Pbx1*, *Pbx2* and *Pbx3* transcripts are found in most fetal and adult tissues except for in lymphoid cells where *Pbx1* is absent (Monica et al., 1991). A comprehensive spatio-temporal expression of mouse *Pbx* genes has been curated and maintained by the gene expression database (GDX) team at the Jackson Laboratory and accessible at Mouse Genome Informatics (MGI) site (http://www.informatics.jax.org/), in which RNA in situ hybridization, immunohistochemistry, Western blot and RT-PCR data are made available (Finger et al., 2011). In zebrafish larvae, *pbx1a*, *pbx2*, *pbx3b*, and *pbx4/lazarus* all show similar expression patterns and are especially active in the central nervous system and retina as revealed by in situ hybridization (http://zfin.org). In *D. melanogaster* at embryonic stage 9 to 10 (equivalent to 220 to 320 min after egg laying), *exd* is expressed in the procephalic ectoderm primordium (includes visual and
central brain primordia), mesoderm and endoderm derivatives. In *C. elegans*, although the expression data are not complete for *ceh-20 (Pbx ortholog)* and its variants, *ceh-40* and *ceh-60*, initial observations suggest that they are expressed in the nervous system and also ubiquitously expressed. For an update of *Pbx* spatio-temporal expression data of *D. melanogaster* and *C. elegans*, visit [http://flybase.org](http://flybase.org) and [http://www.wormbase.org](http://www.wormbase.org), respectively.

Monomeric PBX proteins bind to DNA poorly. Similar to HOX proteins, formation of PBX-HOX heterodimer increases the DNA binding affinity of PBX proteins (see section 1.1.6.). Structural analysis from X-ray crystallography and NMR data shows that the PBX HD is very similar to the HOX HD, i.e. three alpha helices packed around a hydrophobic core. The HOX-PBX HDs bind to DNA in a head-to-tail orientation (Fig. 1.3B). However, upon DNA binding, PBX forms a fourth alpha helix C-terminal to the HD, which packs against helix 1 and helix 3 that is believed to stabilize the homeodomain structure and account for the increased DNA binding affinity. The hydrophobic YPWM/ANW motif of HOX protein binds to the hydrophobic binding pocket in PBX HD formed by interaction between TALE loop connecting helix 1 and helix 2, the C-terminus of helix 3 and the turn between helix 3 and helix 4 (Bourdon et al., 2005; Jabet et al., 1999; Passner et al., 1999; Piper et al., 1999; Sprules et al., 2000, 2003). In addition, PBX proteins have also been shown to form homodimers by interaction with the PBC-B subdomains (Calvo et al., 1999; Neuteboom and Murre, 1997), as well as heterodimers with non-HOX partners belonging to the MEINOX family members requiring PBC-A of PBX and HR1 of MEINOX protein (reviewed in Featherstone, 2003; Moens and Selleri, 2006).

*Pbx1<sup>−/−</sup>* heterozygous mice are fertile and exhibit no gross abnormalities except for smaller body size while homozygous mutants exhibit embryonic lethality (between E15.5 and 16.5), with progressive edema, reduced vascularization, pallor, small thorax and abdomen, and hunched posture, among others features (DiMartino et al., 2001). These homozygotes display patterning defects of the axial and appendicular skeleton, and severe reduction (hypoplastic) of several abdominal and thoracic organs, and aplastic spleen (Selleri et al., 2001). For example, E16 dpc *Pbx1<sup>−/−</sup>* null mice have stunted development of ventral ribs, sternum and clavicles as assessed by Alcian Blue and Alizarin Red staining. The cervical and thoracic vertebrae are irregularly shaped
and stacked, and some are abnormally fused (Selleri et al., 2001). In addition, homozygous mutants showed anterior homeotic transformation of the second branchial arch (BA2) structure to BA1 identity (Selleri et al., 2001). Moreover, \( Pbx1^{-/} \) null mice show reduced chondrocyte proliferation and an accelerated progression of endochondral ossification causing precocious bone formation that affects rib development (Selleri et al., 2001). \( Hox \) and \( Meis \) expression in \( Pbx1^{-/} \) null mice is unaffected (Selleri et al., 2001). \( Pbx1^{-/} \) null mice are also anaemic (with 5% haematocrit as compared to 38% in wild-type) with hypoplastic fetal liver and reduced repopulation capacity of HSC (as assessed by competitive reconstitution assay and radioprotection experiments). Furthermore, the anaemic phenotype of \( Pbx1^{-/} \) mice is associated with reduced clonogenic potentials and proliferation rate of cells important for erythropoiesis (e.g. common myeloid progenitors, megakaryocyte/erythrocyte progenitors) (DiMartino et al., 2001).

1.2.2. MEINOX class proteins: MEIS and PKNOX

\( Meis \) and \( Pknox \) (MEINOX family) are homeobox genes that encode TALE HD containing transcription factors. In human, there are three \( MEIS \) genes (\( MEIS1, MEIS2, MEIS3 \)) which are highly related to the \( PKNOX \) genes (e.g. \( PKNOX1 \) and \( PKNOX2 \); formerly known as \( PREP \) genes). Proteins encoded by these genes share a conserved N-terminal bipartite protein-interaction domain (termed MEINOX), which is composed of HR1 (or MH1) and HR2 (or MH2) sub-domains involved in protein-protein interactions with PBC proteins (Featherstone, 2003; Knoepfler et al., 1997; Mann and Affolter, 1998). MEINOX proteins are closely related the PBC proteins whose PBC domain is evolutionarily related to the MEINOX domain (Bürglin, 1998; Featherstone, 2003).

1.2.2.1. \( Pknox \) genes

Metazoan \( Pknox \) genes are closely related to the plant \( Knotted \) gene (Bürglin, 1998; Mukherjee and Burglin, 2007). \( Pbx/knotted homeobox 1 \) (\( PKNOX1 \); previously known as \( PBX regulating protein, PREP \)) gene was first isolated and cloned through an exon trapping approach during a search for genes on human chromosome 21 (Chen et al.,
Independently, PKNOX1 protein was identified as a component of the multimeric Urokinase Enhancer Factor 3 (UEF3) responsible for the regulation of the plasminogen activator, urokinase (Plau) enhancer containing 5’-TGACAG-3’ core sequence, as well as in other AP-1 regulated promoters (Berthelsen et al., 1998a). UEF3 complexes consist of a common 64 kDa subunit and either 40 kDa or 50 kDa subunit. The 64 kDa subunit was identified as PKNOX1, while the 40 kDa and 50 kDa subunits were PBX1B and PBX2, respectively (Berthelsen et al., 1996; Berthelsen et al., 1998a). The complexing of PKNOX and PBX proteins increases PKNOX binding affinity to 5’ TGACAG 3’ site, which is similar to the binding site of TGIF and MEIS1 (Berthelsen et al., 1998b; Chang et al., 1997), and also to 5’-TGATTGAT-3’ site, which contains PBX binding sites (Berthelsen et al., 1998b). Furthermore, PKNOX1 and PBX protein (either PBX1A or PBX1B) dimerization requires the N-terminal region of PKNOX containing the HR1 and HR2 subdomains and PBC-A domain of PBX. Similar to the PBX-MEIS complex, a PBX-PREP complex can form under DNA-free conditions (Berthelsen et al., 1998a; Berthelsen et al., 1998b). PKNOX1 has been shown to interact with PBX1 and HOXB1 to form a ternary complex on a HOXB1-responsive target, e.g. the mouse Hoxb1-ARE, and enhances the transactivation activity of HOXB1-PBX1 in a reporter assay in cultured cells (Berthelsen et al., 1998b). The DNA binding domain of PKNOX1 (but not HOXB1) is dispensable for the formation of a ternary HOX1B-PBX1-PKNOX1 complex on DNA (Berthelsen et al., 1998b). The evidence for ternary HOX-PBX-PKNOX function in vivo can be drawn from the study of a mouse Hoxb2 enhancer region, where Hoxb2 expression in r4 is dependent on an intact MEIS/PKNOX binding site and HOX-PBX binding site. Importantly, HOXB1-PBX1-PKNOX1 heterotrimer has been shown to bind to this enhancer region (Ferretti et al., 2000). A ternary HOX-PBX-PKNOX complex has also been shown to regulate several HOX target genes, namely rat renin (ren) involved in the renin-angiotensin pathway important for regulating blood pressure and electrolyte balance (Pan et al., 2005) and mouse Nuclear Receptor Subfamily 5, Group A, Member 1 (Nr5a1; previously known as Ad4bp or Sf-1) (Zubair et al., 2006).

In human and mouse, there are two PKNOX/Pknox genes (PKNOX1/Pknox1 and PKNOX2/Pknox2). There are three in zebrafish (pknox1.1, pknox1.2, and pknox2) and two in frog (pknox1 and pknox2). While no Pknox orthologs are found in D.
melanogaster, they are present in the genome of other insects (Mukherjee and Burglin, 2007). In adult mouse, Pknox1 is expressed ubiquitously and more abundantly in testis and thymus (Ferretti et al., 1999). During mouse embryogenesis, Pknox1 is expressed by at least E9.5 days post coitus (dpc) and throughout development until at least E17.5 dpc (Ferretti et al., 1999). In zebrafish, pknox1.1 is maternally expressed and then ubiquitously expressed up to 24 hours post fertilization (hpf), after which it is restricted to the head from 48 hpf onwards (refer to http://zfin.org; Deflorian et al., 2004). Knocking down zebrafish pknox1.1 function by morpholino technology (see section 1.6) produces a phenotype at 5 days post fertilization (dpf) involving smaller heads and eyes, under-developed pectoral fin, missing swim bladder and jaws, among other features. Moreover, pknox1.1 morphants show disrupted hindbrain segmentation (as assessed by genes marking rhombomere borders [pax6a, foxb1a] and hindbrain markers such as pax2a, hoxb1a, hoxa2, hoxb2, and egr2a [aka krox20]), perturbed migration of hindbrain motoneurons, and interrupted pharyngeal chondrogenesis, thus establishing an important developmental role for pknox1.1 in hindbrain development and craniofacial chondrogenesis (Deflorian et al., 2004). In mice, most hypomorphic pknox1<sup>1/2</sup> mutants die between E17.5 and P0 (embryonic lethal), with general organ hypoplasia, smaller body size, anaemia, eye abnormalities (e.g. reduced lens, abnormal retinal lamination and retinal pigment epithelium duplication) and hemorrhages (Ferretti et al., 2006). In the fetal liver of Pknox1<sup>1/2</sup> mice, the number of Pbx1b- and meis1-positive cells show marked reduction as compared to sibling controls (as assessed by immunohistochemistry), and erythrogenesis is perturbed (as assessed by flow cytometry). In the Pknox1<sup>1/2</sup> mice, there is general reduction of the expression of Pax6 and Meis1 in the eye structures, e.g. lens and retina (Ferretti et al., 2006). Nuclear extracts of E10.5 Pknox1<sup>1/2</sup> whole embryos show reduced DNA binding activity to oligonucleotides containing PKNOX1/MEIS1-PBX, PBX-HOX, or PKNOX/MEIS-PBX-HOX ternary binding sites. Moreover, PBX1 and MEIS1 protein levels are reduced in nuclear extracts of Pknox1<sup>1/2</sup> embryos, compared to control (Ferretti et al., 2006).
1.2.2.2. Meis genes

Meis1 (or Myeloid ecotropic viral integration site 1) was first identified as a proto-oncogene causative for the murine myeloid leukemia induced by viral insertional mutation (Moskow et al., 1995). Two other Meis genes, e.g. Meis2 and Meis3 were identified based on sequence similarity (Nakamura et al., 1996; Oulad-Abdelghani et al., 1997). There are three MEIS/Meis family members in human, mouse and frog, six meis family members in zebrafish, i.e. meis1, meis2a, meis2b, meis3, meis4.1a and meis4.1b (http://zfin.org), and one D. melanogaster homolog, hth. In mouse, alternative splicing of Meis1 transcripts produces several transcript variants (and the corresponding protein isoforms), i.e. Meis1a to Meis1d. Meis1a is composed of all 13 exons of Meis1 gene. Meis1b lacks exon number 12 and hence, differs from Meis1a at the C-terminus. Meis1c lacks exon number 6 which corresponds to Val-162 to Gln-210, while Meis1d lacks exon number 8 which corresponds to the HD domain (Bischof et al., 1998; Crist et al., 2011; Geerts et al., 2005; Knoepfler et al., 1997; Wermuth and Buchberg, 2005). Alternative splicing also increases the diversity of mouse Meis2 gene, in which more than four variants, i.e. Meis2a to Meis2d, were identified (Oulad-Abdelghani et al., 1997).

Meis gene expression is found in a variety of tissues derived from all three germ layers during embryogenesis. Their spatiotemporal expression is dynamically regulated. For example, meis1 expression is detected in shield stage zebrafish, as early as 6 hours post fertilization (hpf; Amali et al., 2013); it is subsequently detected in the neural plate and presumptive segmental plate at late gastrula (~8 hpf). During the segmentation period (10 – 24 hpf), meis1 is present predominantly in the neural rod, with spatially restricted expression in the forebrain, midbrain, and hindbrain. Its expression is detected in the eye field at 15 hpf and persists up to 4 days post fertilization (dpf; Bessa et al., 2008). At 24 hpf, meis1 expression is also detected in the olfactory bulb, tectum, hindbrain and spinal cord (section 3.3; http://zfin.org; Bessa et al., 2008; Melvin et al., 2013). Of all Meis genes, meis1 and meis2a are expressed during early development of the eye (Bessa et al., 2008). In X. laevis, meis1 expression is detected in the eye, branchial arches, midbrain, hindbrain, spinal cord, and neural crest at NF stage 25 (27 hpf; Maeda et al., 2002). In mouse, Meis1 and Meis2 are expressed at least E7.0 dpc and up to E17.0 dpc onwards, whereas Meis3 expression...
starts around E11.0 dpc and is subsequently maintained at low levels up to E17.0 dpc (Nakamura et al., 1996). Mouse Meis1, Meis2 and Meis3 are all expressed in the embryonic brain and eye (in situ hybridization results shown in http://www.informatics.jax.org) and in the male and female genital tracts (Williams et al., 2005). Meis1 is also expressed in the ear, endodermal derivatives (e.g. soft tissues of mediastinum and midgut) and spinal cord in E11 dpc mice (refer to Hisa et al., 2004). Also, Meis1 is expressed in HSCs (Sca-1+Lin−) in adult marrow and E14.5 fetal liver, and its expression is downregulated upon hematopoietic differentiation (Pineault et al., 2002). In chick, Meis2 expression is detected in the lateral mesoderm of the trunk and whole limb bud mesenchyme at Hamburger–Hamilton (HH) stage 17. As the limb bud starts to develop, Meis2 (and Meis1) expression becomes restricted to the proximal region of the limb buds at HH stage 20 onwards, which is negatively regulated by the activity of distal factors such as Bmp2, Hoxd11, Hoxd13 among others (Capdevila et al., 1999; Mercader et al., 1999). Similarly, Meis1 expression is detected in the lateral mesoderm during earlier stages (~E9.5 dpc; 22 somites), and when the limb bud starts to develop (e.g. ~E9.5 dpc; 23 to 28 somite stages), its expression is restricted to the proximal region (Mercader et al., 1999; Mercader et al., 2009). The cis-regulatory regions of Meis1 genes in vertebrates are highly conserved and several highly conserved non-coding regions (HCNRs) have been isolated, e.g. one HCNR named HHc2:066650 enhancer, which has Pax6 DNA binding sites, is able to recapitulate Meis1 expression by driving GFP expression in the eye, midbrain and hindbrain in zebrafish. Knocking down pax6 gene function by morpholino(s) (either pax6a or pax6b alone, or in combination) strongly decreases the number of GFP-positive cells in the retina and optic tectum of zebrafish. Likewise, mutating Pax6 binding sites in the enhancer abolishes GFP expression in these tissues (Royo et al., 2012). MEIS1 protein may also auto-regulate its own expression as demonstrated directly by the occupancy of MEIS1, PBX1, and CRTC2 on mouse Meis1 promoter in chromatin immunoprecipitation (ChIP) data (Goh et al., 2009; Goh, 2007; Wang et al., 2014). Several signalling pathways influence Meis expression. In X. laevis meis3 expression in neuroectoderm is positively regulated by Wnt signalling from paraxial mesoderm (Elkouby et al., 2010). In chick, Meis1 and Meis2 expression is activated by retinoic acid (RA) while antagonized by fibroblast growth factor (FGF) in the proximodistal axis of limb (Mercader et al., 2000).
1.2.3. MEIS function

A loss of function approach has demonstrated the role for Meis1 in hematopoiesis and vasculogenesis. For example, in Meis1−/− mutant mice, which are embryonic lethal around E14.5 dpc, these mice show internal hemorrhages in neural tube and trunk regions, pallor and reduced liver size (Azcoitia et al., 2005; Hisa et al., 2004). Also, the mutant mice show abnormal eyes (e.g. smaller lens and partial duplication of the retina) and defects in the brain and other organs (not characterized; Hisa et al., 2004). Reduced liver size is followed by a corresponding reduction in the absolute number of HSCs per mouse, with reduced capacity to produce erythroid and myeloid lineages (Azcoitia et al., 2005; Hisa et al., 2004). In addition, hematopoietic clusters (identified by PECAM+Runx1 or Runx1+CD41 expression) in regions in which definitive HSCs arise (e.g. dorsal aorta of aorta-gonad-mesonephros, AGM) are reduced in number in mutant mice (Azcoitia et al., 2005).

Meis1 is indispensable for megakaryocyte development, and loss of Meis1 function causes a drastic drop of megakaryocyte precursors in fetal liver, as well as circulating megakaryocytes and platelets/thrombocytes (Azcoitia et al., 2005; Hisa et al., 2004). Megakaryocytes are blood cells involved in the production of platelets, which are important for normal hemostasis (or blood clotting/coagulation). Meis1−/− mutant mice show vascular defects as assessed by immunohistochemical staining with endothelial markers PECAM or α-smooth muscle actin (SMA), which display abnormal microvasculature shape and organization (Azcoitia et al., 2005; Hisa et al., 2004). In zebrafish, knocking down the hoxd4a gene has been shown to impair erythropoiesis, vasculogenesis and angiogenesis processes, and this is in part due to downregulation of meis1 gene. Similarly, genes involved in hemangioblast development (e.g. scl1 and lmo2) are reduced in hoxd4a morphants. The hemangioblast is a precursor cell with potential to give rise to hematopoietic and endothelial lineages. Co-injection of meis1 mRNA rescues hematopoiesis and vasculogenesis in hoxd4a morphants (Amali et al., 2013). Similarly, morpholino induced loss-of-function of meis1 in zebrafish has been reported to affect both hematopoiesis and vasculogenesis (Cvejic et al., 2011; Minehata et al., 2008; Pillay et al., 2010).
Axial eye patterning is important for providing positional cues for correct retinotectal connections and this is dependent on the asymmetric expression of several determinants, such as the ephrin ligands (efn) and receptors (eph), which is regulated by multiple signaling pathways. For example, the DV axis of the retina is demarcated by dorsal Efnb-expressing and ventral Ephb-expressing domains, which are regulated by bone morphogenetic protein (Bmp) and Hedgehog, respectively (Erickson et al., 2010). meis1 function has been shown to be important for establishing proper positional information for the eye and tectum. In zebrasfish, knocking down meis1 function perturbs to the efn-eph code required for positional information. For example, meis1 knockdown reduces tbx5 (Bmp target gene) expression in the dorsal retina with concomitant reduction in efnb2a (Tbx5 target gene), whereas ventrally expressing vax2 and ephb2 expand dorsally. Similarly, patterning along the nasal-temporal axis of the retina and tectum is affected in meis1 morphants. Importantly, loss of regional identity in retina and tectum causes retinotectal map defects, e.g. altered RGC axon connection to the tectum (Erickson et al., 2010). Also, MEIS1 has been shown to regulate Pax6 expression during murine lens morphogenesis (Zhang et al., 2002), cell cycle regulators (e.g. cyclin D and c-myc) required for maintenance of retinal progenitor cells (RPCs) (Bessa et al., 2008), and Foxn4 during RPC differentiation (Islam et al., 2013). Knockdown of meis1 in zebrafish has been shown to cause microphthalmia due to reduced proliferation rate of retinal progenitor cells (Bessa et al., 2008). Similarly, Meis2 plays important role in eye development. For example, downregulation of Meis2 expression in chick retina by Shh signaling is important for retinal ganglion cell differentiation (Heine et al., 2009). In Medaka fish (Oryzias latipes), aberrant upregulation of meis2 expression caused by miR-204 knockdown produces fish with eye defect characterized by the presence of microphthalmia, coloboma, eye patterning, and lens defects (Conte et al., 2010). These and several other reports implicate Meis family proteins in the control of retinal progenitor cells proliferations and their downregulation coincide with retinal cell differentiation (Bessa et al., 2008; Conte et al., 2010; Erickson et al., 2010; Heine et al., 2009).

Meis1 is important for the proper patterning of hindbrain similar to Pbx function. For example, disrupting meis1 function in zebrafish by injection of mRNA encoding dominant negative Meis1 protein caused a reduction in krox20 expression in r3 and r5, resembling lazarus (pbx4) mutant (Waskiewicz et al., 2001). In addition, inhibition of
meis1 activity in lazarus embryos potentiated lazarus phenotype further such that these embryos expressed krox20 at levels much more lower than uninjected lazarus mutants (Waskiewicz et al., 2001). Conversely, wild type meis1 injection into lazarus mutant embryos rescued its phenotype (Waskiewicz et al., 2001). Co-expression of Meis1 and Lazarus (Pbx4) proteins in zebrafish embryo increased the stability of these proteins. This is accomplished by protein-protein interaction as deletion of the N-termini of both proteins required for heterodimer formation abolished the reciprocal stabilization effect (Waskiewicz et al., 2001).

Meis1 is important during limb bud development. Chick limb buds overexpressing retrovirally delivered Meis1 display altered distal limb structures involving the zeugopod and autopod, such that both structures show reduction in size and malformation of skeletal pattern in the limb, e.g. anterior tibia extended continuously through the ankle and displaced autopod structures (Mercader et al., 1999). Proximalization of autopod structures are evidenced by altered skin elements. In wild-type, the skin can be distinguished in the proximal-distal axis by its characteristic skin elements, e.g. feathers on the stylopod and zeugopod but scales on the autopod. In infected limbs, feathers from the zeugopod encroach into the autopod region (Mercader et al., 1999).

The role of Meis1 in cell proliferation has been extensively studied in the context of leukemogenesis. Its activity is aberrantly increased in most acute myeloid- and mixed-lineage leukaemia (AML and MLL, respectively; Wang et al., 2005; Zeisig et al., 2004). Chromosomal translocation of Mixed lineage leukemia (MLL) that fuses N-terminal region of MLL to one of >40 C-terminal fusion partners has been implicated in these cancers, in which all types of MLL fusion-induced leukemias are known to affect Hox gene expression. Moreover, Hoxa9 and Meis1 gene activity are frequently active in these cancers, while co-expression of these genes in mouse bone marrow cells recapitulates and accelerates MLL-ENL-induced immortalization of myeloid progenitors (reviewed in Arigopouloos et al., 2007). Meis1 oncogenic activity requires interaction with HOX and/or PBX proteins (Argiropoulos et al., 2007; Schnabel et al., 2000). Interest in how these interactions affect the transcriptome of cancer cells has been fuelled by the prospect of using this knowledge for therapeutic intervention as well as understanding the biology of stem cell self-renewal (Argiropoulos et al., 2007).
In a different cellular context, instead of promoting cell proliferation, Meis1 function has been shown to negatively regulate cell proliferation. For instance, the proliferation of murine post-natal cardiomyocytes has been demonstrated to be inhibited by Meis1 overexpression and this is accomplished by upregulation of cell cycle inhibitors such as \( p15 \) (aka \( cdkn2b \)), \( p16 \) (aka \( cdkn2a \)) and \( p21 \) (aka \( cdkn1a \)) (Mahmoud et al., 2013).

1.3. \( Creb \) genes

Cyclic adenosine monophosphate response element binding protein (CREB), cAMP responsive element modulators (CREMs) and activating transcription factor (ATFs) are \( Creb \) family members belonging to the basic leucine zipper transcription factor superfamily that also includes the \( c-Fos/c-Jun \) family. CREB proteins are responsive to a variety of extracellular stimuli by acting downstream of second messenger cAMP signaling and cAMP-dependent PKA, to regulate gene transcription via cAMP response elements (CRE), and its activity is mediated by several distinct protein domains. For example, transcriptional activation domain (TAD) is situated at the N-terminal of the protein, and it is composed of three regions, namely a central kinase-inducible-domain (KID) flanked by glutamine-rich Q1 and Q2 domains. At the C-terminal lies the basic leucine zipper (bZIP) domain.

CREB activation is dependent on phosphorylation of Ser133 within KID by PKA, calcium/calmodulin-dependent protein kinase (CaMK) II and IV, protein kinase C (PKC), Ribosomal Protein S6 Kinase, 90kDa, Polypeptide (RPS6KA, formerly known as RSK), mitogen-activated protein kinase-activated protein kinase (MAPKAPK), V-Akt Murine Thymoma Viral Oncogene Homolog (AKT), and mitogen- and stress-activated kinases (MSK) (de Groot et al., 1993; Deak et al., 1998; Du and Montminy, 1998; Ginty et al., 1994; Gonzalez and Montminy, 1989; Shaywitz and Greenberg, 1999; Sun et al., 1994; Tan et al., 1996; Xing et al., 1996). Phosphorylation of Ser133 promotes KID interaction with KID-interacting (KIX) domain of coactivator CBP and p300 (Parker et al., 1996). KID also contains a region for ataxia-telangiectasia-mutated (ATM) and casein kinases (CKs) phosphorylation site termed ATM/CK (e.g. Ser108, Ser111, Ser114, Ser117, and Ser121 in CREB1B), in which CREB/ATF is rendered inactive when these sites are hyperphosphorylated.
during cellular response to DNA damage (Shanware et al., 2007; Shanware et al., 2010). For example, hyperphosphorylated ATF1 induced by ionizing radiation showed a 4-fold reduced affinity for CBP (Shanware et al., 2010). There are other phosphorylation sites in CREB, which evoke context-specific effects on CREB transactivation, e.g. Ser142 phosphorylation by CaMKII has been shown to inactivate CREB by inducing dissociation of its dimer, which in turn abrogates CBP recruitment (Kornhauser et al., 2002; Wu and McMurray, 2001). On the contrary, through the use of Ser142Ala knock-in mice, light stimulus-induced phosphorylation of Ser142 has been shown to impart CREB transcriptional activity during the regulation of circadian rhythm in the suprachiasmatic nucleus (SCN) (Gau et al., 2002). The CREB phosphorylation state at Ser133 is regulated by a balanced activity of kinases and phosphatases such as Ser/Thr protein phosphatases type 1 and 2A (i.e. PP1 and PP2A) (Alberts et al., 1994; Wadzinski et al., 1993). In hippocampal neurons, synaptic activity that leads to phosphorylation and maintenance of the phosphorylated state at Ser133 of CREB usually requires concomitant inhibition of phosphatases such as PP1 (Bito et al., 1996).

The bZIP domain is largely alpha helical and is composed of a dimerization region and DNA-binding basic region, which is conserved among CREB members. CREB members are able to form homo- and heterodimers among themselves, such as with ATF-1 (Hurst et al., 1991), CREM (Foulkes et al., 1991), and they can also form heterodimers with other bZIP family, like the c-Fos/c-Jun family members (Benbrook and Jones, 1990; van Dam et al., 1993; Vinson et al., 2002). Such combinatorial interaction can provide bZIP members with a sophisticated response to stimuli. In addition, CREB bZIP basic region binds to cAMP response element (CRE) consensus sequence 5’-TGACGTCA-3’ and half CRE 5’-CGTCA-3’, while Q1 and Q2 regions are necessary for interaction with TATA-binding protein-associated factor II 135 (TAFII135) and recruitment of transcription machinery (Felinski and Quinn, 2001). In addition, CREB can also function as transcription activator independent of Ser133 phosphorylation and CBP recruitment by interaction with coactivator CRTC family of proteins via an association with the bZIP DNA-binding domain of CREB and N-terminal coiled-coil domain of CRTC proteins (Conkright et al., 2003).
The Creb gene contains at least 11 exons and alternative splicing produces a variety of CREB isoforms (Walker et al., 1996). In rat testis, it has been shown that alternatively spliced exons produce CREB isoforms with different activities, e.g. either as transcriptional activators or repressors which lacked the N-terminal TAD containing KID (Walker et al., 1996). There are three major CREB isoforms, e.g. alpha (α), beta (β) and delta (Δ), all of which have the three protein domains essential for transactivation function, namely Q domain, KID domain, and bZIP domain (Blendy et al., 1996; Ruppert et al., 1992). CREBα and CREBΔ differ only with respect to the presence or absence of α domain derived from exon 5, whereas CREBβ lacks α domain and have shorter Q1 domain (exon 2; see Fig. 1.5) (Blendy et al., 1996).

Figure 1.5  The structure of mouse Creb gene

(A) There are 11 exons depicted as open boxes and introns as lines (not to scale); (B) three major Creb isoforms due to alternative splicing are shown, i.e. CREBΔ, α, and β. Exons encoding Creb protein domains such as Q1 and Q2 (glutamine-rich domains), KID = kinase-inducible domain, b = basic motif, and ZIP = leucine zipper motif are indicated (adapted from Blendy et al., 1996).

CREB is a versatile transcription factor that plays important function in cell proliferation, neuronal survival and differentiation, formation of memory, synaptic plasticity, metabolic regulation, and longevity, among others. CREB function in cell proliferation have been linked to leukemia, in which increased expression of CREB gene was detected in bone marrow cells of patients with acute lymphoid or myeloid cells (Crans-Vargas et al., 2002; Kinjo et al., 2005). Moreover, ectopic expression of CREB under hMRP8 promoter which drives expression in myeloid cells has been shown to enhance proliferation of stably transfected myeloid cell lines with
concomitant increase in cyclin A1 expression (Kinjo et al., 2005). When these cells were induced to differentiate along the erythroid lineage by using sodium butyrate, CREB expressing myeloid cells showed reduced differentiation activity (Kinjo et al., 2005). CREB function has also been shown to be required in development of acute leukemia mediated by the activity of MLL fusion proteins and HOX-PBX-MEIS complexes (Wang et al., 2010). For example, co-expression of CREB and MLL-AF6 shortened the latency period for the development of leukemia in transplanted mice, indicating the role of CREB in promoting leukemic cell proliferation. Conversely, forced expression of dominant negative CREB mutant proteins decreased the colony forming ability of the leukemia cells (Wang et al., 2010). In transgenic mice that overexpress Creb in bone marrow cells, these mice developed myeloproliferative syndrome with enlarged spleen (Kinjo et al., 2005; Shankar et al., 2005).

The role of Creb in memory was first unveiled in the study of the sea slug Aplysia, in which 5-hydroxytryptamine induction of long-term facilitation of the gill withdrawal, was also shown to activate reporter gene driven by CREs (Kaang et al., 1993). Subsequent experiments in D. melanogaster showed that disrupting CREB function blocked long-term memory formation (Yin et al., 1994). Likewise, targeted disruption of CREBα and CREBΔ activity in mice affected long-term memory formation as assessed by their performance on fear conditioning test comprising a Morris water maze task (Bourtchuladze et al., 1994). CREB has been shown to be involved in other associative learning processes (i.e. adaptive processes that permit model organisms to learn to anticipate events) as well, e.g. cued/contextual fear memory, conditioned taste aversion memory, and olfactory memory (Bourtchuladze et al., 1994; Graves et al., 2002; Kogan et al., 1997; Lamprecht et al., 1997; Pittenger et al., 2002), while one group found no significant link between CREB and memory in these loss-of-function models (Balschun et al., 2003). The discrepancy is believed to be due to functional redundancy of other CREB proteins (reviewed in Sakamoto et al., 2011). However, in a converse experiment in which CREB function is constitutively active (VP16-CREB chimera) and conditionally expressed in transgenic mice (e.g. tTA/tetO system), the expression of constitutively active CREB in postsynaptic neurons of the Schaffer collateral pathway has been shown to aid in the formation long-lasting long term potentiation (LTP) and to lower the threshold for single tetanic train to achieve late-phase LTP (Barco et al., 2002). Similarly, injection of phospho-
CREB into the sensory neurons of *Aplysia* allowed production of serotonin-stimulated long term facilitation that last for several days (Casadio et al., 1999). Although the mechanisms underlying the ability of CREB to facilitate memory are not completely understood, we know that memory formation could be attributed to the generation of more or new synapses (important for plasticity and memory consolidation). Interestingly, expression of constitutively active CREB or CaMKIV in hippocampal pyramidal cells has been shown to increase the number of “silent synapses” (Marie et al., 2005), which have been shown to be crucial substrate for long term memory (reviewed in Atwood and Wojtowicz, 1999; Kerchner and Nicoll, 2008).

CREB has been shown to mediate the activity of neuronal prosurvival factor (e.g. nerve growth factor [NGF] and neurotrophins) by activating CREB-dependent gene expression important for neuronal survival such as *Bcl-2* (Riccio et al., 1999). Several inhibitors can be used to suppress CREB activity, e.g. ACREB which form non-DNA binding dimer with endogenous CREB and hence isolate the complex from CRE sequences, while Ser133-unphosphorylatable and inactive CREBM1 competes with endogenous CREB to bind to DNA target sites. Inhibition of CREB activity by intranuclear microinjection of expression vector encoding CREB inhibitors (e.g. A-CREB or CREBM1) caused apoptotic death to sympathetic neurons cultured in the presence of NGF, whereas injection of wild type showed no effect (Ricchio et al., 1999). In contrast, injection of constitutively active *Creb* to these neurons promoted neuronal survival in the absence of NGF, whereas control neurons underwent apoptotic cell death within 36 to 49 hours after NGF withdrawal (Ricchio et al., 1999). The loss of neurons could be a reason for reduced brain size in *Creb*−/− mice (Rudolph et al., 1998).

The role of *Creb* in energy balance can be illustrated by its regulatory effect on genes responsible for metabolic processes. For example, CREB has been shown to stimulate the expression of rate-limiting enzyme *phosphoenolpyruvate carboxykinase* (*PEPCK*) required during liver gluconeogenesis (Liu et al., 1991; Quinn and Granner, 1990). In order to study the role of *Creb* in gluconeogenesis, Herzig et al. (2001) generated mice expressing dominant negative ACREB specifically in liver cells (driven by liver-specific albumin promoter/enhancer). These mice were hypoglycaemic and showed a reduction in *PEPCK* and *Glucose-6-Phosphatase* (*G6PC*) mRNA levels in the liver (Herzig et al., 2001). In another approach, systemic injection
of adenovirus expressing ACREB in mice was used. This approach produced 80-90% infected liver cells as assessed by fluorescence from co-expressed reporter gene. ACREB infected mice showed comparable blood sugar level under fed state, but this level dropped drastically under fasting condition, hence suggesting that functional CREB is required for gluconeogenesis during fasting (Herzig et al., 2001). Several genes required for gluconeogenesis were also downregulated in ACREB infected mice, e.g. PEPCK, G6PC, and pyruvate carboxylase (PC). While PEPCK has been shown to be directly targeted by CREB, the activation of other gluconeogenic genes was shown to be mediated by Peroxisome Proliferator-Activated Receptor Gamma, Coactivator-Related 1 (PPRC1, formerly known as PGC-1). Interestingly, PPRC1 is a direct target of CREB protein, which prompts the author to suggest a two-step model for the activation of gluconeogenic genes. In the first step, upon stimulation by catecholamine and glucagon, CREB is activated by Ser133 phosphorylation and this leads to induction of CRE-containing gluconeogenic genes (e.g. PEPCK). During prolonged fasting, CREB proceeds to induce PPRC1 expression and initiate gluconeogenesis under glucocorticoid signaling (Herzig et al., 2001).

Creb has been implicated in other developmental processes. For instance, Creb function has been linked to aging in worms. Inactivation of either Creb (crh-1) or Crtc1 (crtc-1) function in C. elegans has been shown to favour longevity with concomitant upregulation of ER stress response genes (Mair et al., 2011). Atf2 (formerly Creb2), which is a Creb family member, has been shown to be important in eye development. Atf2 deficient homozygous mice display microphthalmia which was caused by loss of lens tissue due to p53-dependent apoptotic event initiated at E14.5 dpc (Hettmann et al., 2000). The role of Creb in myogenesis was deduced from the fact that spatial regulation of CREB Ser133 phosphorylation during myogenesis corresponded to myogenic region in mice where Pax3, Myf5 and Myod1 were expressed (Chen et al., 2005). This was again demonstrated in Creb+/– mice which displayed myogenic defect as assessed by the reduced expression of PAX3, MYF5 and MYOD1 proteins in muscle tissues. The effect was more pronounced when adenovirus expressing ACREB was used to inactivate all Creb family members including ATF and CREM, in which PAX3, MYF5 and MYOD1 expression was dramatically reduced in muscle tissues where the injection took place (Chen et al., 2005). Subsequent experiments revealed that these myogenic genes are regulated by WNT
signaling and mediated by the cAMP-PKA-CREB pathway (Chen et al., 2005). CREB has also been shown to regulate the development of somatotroph in the pituitary. Somatotrophs are pituitary cells responsible for growth hormone secretion in response to signals from the hypothalamus. Mice with targeted inactivation of CREB function in somatotroph produced offspring with stunted growth (Struthers et al., 1991).

1.4. Crtc genes

The gene coding for cAMP response element (CRE) binding protein (CREB)-regulated transcription coactivator (CRTC1) was the first member of the CRTC gene family discovered in a size-fractionated human brain cDNA library (Ishikawa et al., 1998). This provided the necessary information to uncover other family members based on sequence homology (Conkright et al., 2003; Iourgenko et al., 2003). Interest in CRTC function first arise from the chromosomal anomaly that causes human malignant salivary gland tumour, a condition known as mucoepidermoid carcinoma (Martins et al., 2004; Tonon et al., 2003), which is due to a t(11;19) translocation that produced N-terminal Crtc1 fusion to C-terminal transactivation domain (TAD) of MAML2. Oncogenic activity of CRTC1-MAML2 (formerly known as MECT1-MAML2) was thought to derive from disruptive Notch signaling per se (Tonon et al., 2003), considering that Notch signal is implicated in cancer (Jhappan et al., 1992). However, the molecular mechanism was unknown, and complicated by the fact that CRTC1-MAML2 is unable to effect Notch signaling. The answer came during a search for modulators of CREB activity by high-throughput screening of human cDNA library by CRE luciferase reporter assay. CRTC1 and CRTC2 were among the proteins found to potentiate the reporter activity (Conkright et al., 2003). Their interaction with CREB1 is mediated by the N-terminal coiled coil domain of CRTC proteins and the bZIP of CREB, without affecting CREB DNA binding activity and independent of the kinase inducible domain (KID). Interestingly, CREB activates Notch target gene HES-1 implicated in cancer caused by CRTC1-MAML2, and this gene contains CRE consensus site in its promoter; and activation of HES-1 by the fusion protein is lost in the presence of dominant negative CREB (A-CREB; Conkright et al., 2003). These observations provided the clue that CREB and CRTC1-MAML2 are involved in the deregulation of NOTCH target genes (as well as CREB
target genes) in mucoepidermoid carcinoma (Conkright et al., 2003; Coxon et al., 2005; Wu et al., 2005). Subsequent observations supported this contention, namely the proliferative and transforming activity of CRTC1-MAML2 is removed when CREB activity is disrupted (Wu et al., 2005). Furthermore, CREB role in cancer has been reported, thus suggesting that tumorigenic potential of CREB could be mediated by CRTC1 (Cho et al., 2011; Gu et al., 2011; Jean et al., 2000).

In vertebrates such as human, mouse and zebrafish, there are three CRTC genes (CRTC1, CRTC2 and CRTC3). CRTC1 in humans and mouse is ubiquitously expressed in adult tissues, with more abundant expression in the brain tissue as assessed by microarray analysis (http://biogps.org; Conkright et al., 2003; Wu et al., 2013). Worm crtc-1 presents the same spatial expression profile (Mair et al., 2011). In D. melanogaster, crtc is maternally deposited and its expression is ubiquitous in later stages of development up to embryonic stage 10 (http://flybase.org). In comparison to CRTC1, CRTC2 and CRTC3 are more abundantly expressed in peripheral tissues (Conkright et al., 2003). As for functional role, Crtc1 has been shown to be important in development of the long-term memory in mice and fly in brain region analogous to human hippocampus (Hirano et al., 2013; Sekeres et al., 2012) and longevity in C. elegans (Mair et al., 2011). Crtc2 has been implicated in maintenance of energy homeostasis and metabolism (Altarejos and Montminy, 2011; Lerner et al., 2009), maintenance of the islet of Langerhans cells (Eberhard et al., 2013). Crtc3 is also involved in regulating energy balance (Song et al., 2010). Among the Crtc genes, Crtc1 has been shown to play a predominant role in brain function.

The CRTC1 protein is composed of an N-terminal region highly conserved in Crtc family members, which can fold into a single continuous 28 residues helix (and not a previously thought coiled coil structure) that can interact with the DNA-binding basic leucine zipper (bZIP) domain of CREB (Luo et al., 2012). Downstream of this CREB-binding domain (CBP) lies one nuclear localization sequence (NLS) and two nuclear export sequences (NES), which are also conserved in other CRTC proteins (Screaton et al., 2004). Post-translational modifications of residues in the vicinity of these transport recognition sequences are important for CRTC1 subcellular localization. For example, phosphorylation at Ser151 by AMPK/SIK2 promotes 14-3-3 binding and sequestration of CRTC1 to the cytoplasm (for degradation), whereas
dephosphorylation by calcineurin translocates the protein into the nucleus (Bittinger et al., 2004; Dentin et al., 2007; Katoh et al., 2006). In addition, competitive glycosylation at these phosphorylation sites by O-linked GlcNAc transferase (OGT) promotes CRTC nuclear localization (Dentin et al., 2008). Moreover, proline isomerization at phosphorylated serine-proline (pSer136-Pro) of CRTC2 by PIN1 leads to its cytoplasmic localization (Nakatsu et al., 2010).

CRTC2 has been shown to regulate RNA splicing and this activity has been shown to depend on the splicing domain near to the C-terminal TAD. However, the mechanistic basis of this function is currently unknown (Amelio et al., 2009). The function of TAD domain has been demonstrated to coordinate the assembly of the transcriptional apparatus with subunit of TFIID (Conkright et al., 2003; Iourgenko et al., 2003).

CRTC1 is activated by calcium mainly through the Ca2+/Calcineurin pathway. Calcineurin (CaN) is a Ca2+-calmodulin activated Ser/Thr phosphatase that has been shown to remove inhibitory phosphates from CRTC1, and hence relieving inhibition by 14-3-3 that prevented CRTC1 translocation to the nucleus (Screaton et al., 2004). CRTC1 is phosphorylated by energy sensing kinases such as AMPK and SIK2 (Altarejos and Montminy, 2011). cAMP-PKA signaling positively regulate CRTC1 activity, partly by inactivation of SIK2 via phosphorylation and cytoplasmic sequestration in 14-3-3 dependent manner (Henriksson et al., 2012). In addition, glucose can activate CRTC function through α-glycosylation at Ser residues and render CRTC nuclear (Dentin et al., 2008).

CRTC1 interacts with CREB family members (Mair et al., 2011; Mayr and Montminy, 2001; Xiao et al., 2010) and c-Jun/c-Fos (Canettieri et al., 2009) belonging to the bZIP transcription factor family. These family members are involved in the transcriptional network regulated by CREB and AP-1 complex. Interaction with CRTC1 increases CREB members’ transcriptional activity, and increases association of Creb binding protein (CBP) to the complex (Ravnskjaer et al., 2007; Xu et al., 2007). Nuclear CRTC2 in association with CBP is found to undergo protein acetylation that stabilizes the protein by blocking ubiquitin-mediated proteolysis (Liu et al., 2008). In addition, CRTC1 is also a coactivator for MEIS1 proteins. Their
interaction is mediated by CRTC1 N-terminal domain (Goh et al., 2009). CREB and MEIS1 form higher order protein complex with CRTC1 and CBP which is required for the expression of MLL genes, and this interaction is dependent on Glycogen synthase kinase 3 (GSK3) activity (Wang et al., 2010).

1.5. **Crtc1 role in development**

CRTC1 plays an extensive role in development, mainly because of its function as coactivator to CREB members and recently, to MEIS family – hence placing itself as downstream effector to two huge transcriptional networks, which have diverse role in development that covers patterning, growth, energy balance, survival, death and many more. Crtc1 is involved in the regulation of appetite by acting downstream of leptin signal. Leptin produced by white adipose tissue (after a meal) induces nuclear localization of hypothalamic CRTC1 and activation of “satiety” genes *cocaine and amphetamine–regulated transcript prepropeptide* (*Cartpt*) and *KiSS-1 metastasis-suppressor* (*Kiss*) in collaboration with CREB1. Disrupting Crtc1 function produces hyperphagic fat mice (Altarejos et al., 2008). Next, CRTC1 and other CRTC proteins are involved in mitochondria biogenesis and function by cooperating with CREB in the activation of *proliferator-activated receptor gamma coactivator 1α* (*PGC-1α*) expression (Wu et al., 2006); *PGC-1α* is also involved in gluconeogenic program (Herzig et al., 2001). CRTC1 involvement in modulating inflammation through activation of *interleukin 8* (*IL8*) is by cooperative function with CREB protein (Iourgenko et al., 2003). CREB and CRTC1 involvement in long term memory has been shown to involve expression of brain-derived neurotrophic factor (BDNF) required in promoting survival of neurons during memory consolidation (Kovács et al., 2007). Crtc2 and Creb promote gluconeogenesis in liver. During fasting, glucagon promotes CRTC2 dephosphorylation and nuclear localization, and occupancy at promoters of gluconeogenic genes such as *phosphoenol pyruvate carboxykinase* (*PEPCK*), *PGC-1α* and *glucose-6-phosphatase* (*G6Pase*). After refeeding, CRTC2 activity is inhibited by insulin via SIK2/AMPK phosphorylation and ubiquitin-proteasomal degradation (Cheng and Saltiel, 2006; Dentin et al., 2007; Koo et al., 2005). Deficient Crtc function in worm is shown to increase lifespan, by shutting down gluconeogenic program and elevating ER stress response (Mair et al., 2011).
1.6. Zebrafish as a model organism

The popularity of zebrafish (*D. rerio*) as a vertebrate model system for developmental biologists is due to its many useful attributes, but most captivating would be the relative ease of embryonic and genetic manipulation. Zebrafish are oviparous. Their embryos are easily collected and their optical transparency provides unrivalled access to visualization of internal structures. Moreover, embryos can be observed in real-time and a large swath of development can be monitored in a relatively short duration, especially considering that zebrafish embryos have already developed most major organs (Kimmel et al., 1995) and exhibited adult-like behaviour (e.g. touch or escape responses) in just under 72 hpf (Brustein et al., 2003; Granato et al., 1996) as compared to ~3 weeks in mice. In addition, zebrafish are highly fecund and produce abundant eggs from a single mating (e.g. 100 to 200 fertilized eggs/mating) and have a relatively short generation time (e.g. 3 to 4 months) which is useful for the fast production and maintenance of zebrafish transgenic and mutant lines. In terms of maintenance cost, zebrafish facilities are relatively inexpensive and can accommodate more fishes per area than other vertebrate model systems, such as *X. laevis*, chick and mice. Several papers have discussed the usefulness of zebrafish as a vertebrate model system by comparison of zebrafish biology with that of human (Lieschke and Currie, 2007; Menke et al., 2011).

Several forward- and reverse-genetic approaches have been developed to facilitate the studies of gene function in zebrafish, and the resultant mutant libraries have been made available such as the Zebrafish Mutagenesis Project ([http://www.sanger.ac.uk/Projects/D_rerio/zmp/](http://www.sanger.ac.uk/Projects/D_rerio/zmp/)) and the Zebrafish TILLING project ([http://webapps.fhcrc.org/science/tilling/index.php](http://webapps.fhcrc.org/science/tilling/index.php)) (Varshney and Burgess, 2014). Furthermore, gene functional studies in zebrafish have benefited from the recent appearance of targeted mutagenesis tools, such as zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short sequences (CRISPR) (reviewed in Lieschke and Currie, 2007; Varshney and Burgess, 2014). Moreover, transgenic zebrafish reporter lines expressing fluorescent proteins in particular tissues or organs can be used in chemical, forward- and reverse-genetic phenotypic screening, an approach that has been instrumental in gene functional studies (Lieschke and Currie, 2007).
In addition, there is an extensive literature for reference (as of Jan 2014, there are >19,000 publications available in ZFIN), a centralized fish database involving Zebrafish International Resource Center (http://zebrafish.org) and ZFIN (http://zfin.org) (Bradford et al., 2011), and a completed zebrafish genome DNA sequence with genome assemblies reaching the 10th version (i.e. GRCz10) will be updated in early 2014 and maintained by Genome Reference Consortium (GRC) (http://www.sanger.ac.uk/resources/zebrafish/genomeproject.html; http://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/).

1.7. Morpholino knockdown

Morpholino antisense oligo (or MO, and interchangeably “blocker”) is a short chain of about 25 MO-subunits, with each subunit composed of a base, a morpholine ring, and a non-ionic phosphorodiamidate backbone (in place of phosphate group). MO can be divided into two types based on their mode of action, either a splice MO (splMO) or translation MO (tMO). MO-mediated knockdown of RNA targets are not through RNA degradation pathway, but rather via the formation of an RNase H-independent steric blocking mechanism – facilitated by highly specific base-complementation of MO to their target, higher stability, and RNase-resistant property. For translation blockers, they form steric hindrance at the 5’ untranslated region (UTR) of targets in cytosol which prevent the translation machinery function. Similarly, splMO form blockage at splice junctions (or regulatory sites) of pre-mRNA and compete with the spliceosome to disrupt normal splicing. This can also be used against microRNA (miRNA) maturation, ribozyme inhibition, masking RNA translocation sequences, among others (http://www.gene-tools.com/). In terms of the effect on protein product, splMOs produce mutant polypeptides with truncation, domain deletion, and frame-shift mutations, among others, whereas tMOs hamper protein synthesis and reduce the amount of the target protein in the cell. In addition, splice blockers can only act on zygotic mRNA, whereas translation blockers act on both maternal and zygotic mRNAs.

The utility of MO antisense oligo in zebrafish has been demonstrated (Nasevicius and Ekker, 2000). The effectiveness of the “knockdown” was observed from maternal through post-segmentation and organogenesis development stages. The
efficacy (or perdurance) of MO has been reported to last up to 5 dpf (reviewed in Bill et al., 2009). The knockdowns were able to phenocopy most of the previously reported mutant fish strains. However, a caveat regarding this technology (and other knockdown technology) is that MOs can cause off-target effects mediated by induced delta113 \( p53 \) expression and associated cell death (Nasevicius and Ekker, 2000; Robu et al., 2007), and proper control experiments (e.g. non-overlapping MO and RNA rescues) are needed to distinguish \textit{bona fide} effects (Bill et al., 2009; Eisen and Smith, 2008; Nasevicius and Ekker, 2000).

1.8. Research objectives

Previously, we have demonstrated that CRTC1 is a coactivator of MEIS1 in a cAMP-PKA pathway with potential to drive HOX/PBX/MEIS target genes. We speculated that there is a probable involvement of MEIS/CREB complex in the regulation of genes involved in leukemogenesis. This is considering the fact that MEIS and CREB binding sites are present in AML gene FLTR. Moreover, CRTC1 (as well as CBP) served as a common coactivator for both MEIS and CREB, and hence could act as a bridge for MEIS-CREB binding (Goh et al., 2009; Huang et al., 2005). Subsequently, Wang et al. (2010) demonstrated that MEIS1 activity in MLL is indeed dependent on CRTC1 and CREB for activity and for the recruitment of CBP, and that GSK3 phosphorylation of CREB is required in this process.

For this thesis, my main interest is to study the cooperative function of Meis1 and Crtc1 during zebrafish development. In the knockdown study of \textit{meis1} function in zebrafish, several labs have demonstrated the diverse role of \textit{meis1} in the regulation of cell proliferation, patterning, vasculogenesis, and hematopoiesis (Amali et al., 2013; Erickson et al., 2010; Hisa et al., 2004), but so far nothing has been reported for \textit{crtc1} role in fish.

Hypothesis:

(1) We have shown earlier that knocking down \textit{CRTC2} with shRNA dramatically reduced Meis1 transcriptional activity on luciferase reporter gene. It is hypothesized that knocking down \textit{crtc1} gene in zebrafish could also affect \textit{meis1} function.
(2) By the same token, deficient crtc1 could also disrupt other transcriptional networks requiring Crtc1 protein as coactivator.

(3) The cooperative function of Crtc1 and Meis1 is restricted to regions of their co-expression.

Specific aims:

(1) To characterize the two crtc1 genes in zebrafish, namely crtc1 and crtc1b, in which crtc1 is deduced from automated computational analysis whereas crtc1b is derived from a cDNA clone.

(2) To determine the spatiotemporal expression of crtc1 genes in zebrafish, and comparing their expression domains with that of meis1.

(3) To study the role of Crtc1 during zebrafish development by reverse genetics approach using morpholino-mediated gene knockdown technology.
CHAPTER 2 : MATERIALS & METHODS

2.1. Plasmid constructs

2.1.1. Plasmids used in in situ hybridization (ISH) staining

DNA plasmids for synthesizing 
\textit{ascl1b, neurod4, nkd2.2a, sox10}, and \textit{plp1b} RNA probes were a generous gift from Vincent Cunliffe. DNA plasmids for synthesizing \textit{olig2} and \textit{islet1} RNA probes were a generous gift from Bernard Thisse and Christine Thisse. DNA plasmids for synthesizing \textit{crtc1} (Gene Accession no. \textit{xm}_003199425) and \textit{crtc1b} (Gene Accession no. \textit{nm}_001077457) RNA probes were constructed by Looi (2012). DNA plasmid for synthesizing \textit{meis1} RNA probe was described in Amali et al. (2013). DNA plasmid for synthesizing \textit{shha, ihhb, ptch2, and bmp4} RNA probes were a generous gift from Phil Ingham. DNA plasmids for synthesizing \textit{myod1} and \textit{krox20} RNA probes were courtesy of Andrew J. Waskiewicz.

\textit{pST} is an empty cloning vector for the construction of DNA constructs used in the synthesis of ISH RNA probes. \textit{pST} was constructed by inserting SP6 promoter, multiple cloning site (MCS) and T7 promoter containing sequence into \textit{Pfu} DNA polymerase-blunted HindIII and EcoRI restriction sites in \textit{pUC19} (Appendix 4).

Zebrafish \textit{meis2b} (\textit{NM}_130910) was PCR amplified using forward primer \textit{PR93} (5’-\textit{AATGGGGATGAACATGGGTA-3’}) and reverse primer \textit{PR94} (5’-\textit{AATGGGGATGAACATGGGTA-3’}), while \textit{gsc} (\textit{NM}_131017) was with forward primer \textit{PR112} (5’-\textit{GAGACGACACCGAACCATTT-3’}) and reverse primer \textit{PR110} (5’-\textit{CCTCTGACGACGACCTTTTC-3’}). PCR was performed with cDNA template synthesized from 48 hpf larvae. The amplicons were then cloned into \textit{pST} vector. \textit{cyp26a1} (\textit{NM}_131146) was PCR amplified with forward primer \textit{PR75} (5’-\textit{TTTCTTTTTGCGATCGTT-3’}) and reverse primer \textit{PR76} (5’-\textit{TGTACAAAGCTCGGGTTAGG-3’}) from the abovementioned cDNA and T/A cloned into \textit{pCR4 TOPO} (Invitrogen). These constructs are designated as \textit{pST meis2b, pST gsc, and p4T cyp26a1}, respectively (Appendix 4).
2.1.2. Plasmids used in rescue experiment

Zebrafish cDNA encoding Crtc1-flag was linearized from pcDNA3.1 Crtc1-flag (see section 2.1.3) and sub-cloned into pSP64 Poly(A) vector (Promega, P1241) via HindIII and XbaI sites. The above construct is designated as pSP zCrtc1-flag. Morpholino-resistant Crtc1 was PCR amplified using forward primer PR70 (5’-agcaagcttAACATGGCTAGTAGCAACAAATCCAACgaaattcagcg-3’; cap letters denote MO binding site, underscored letters represents mutagenized region) with several bases mutated (see Fig 3.20) at the crtc1 tMO1 binding site (see section 2.4.3) and reverse primer PR69 (5’-AGCTCTAGATATCCAGATCCATGCcGAAAGGT-3’). The amplicon was linearized with HindIII and EcoRV, which was subsequently cloned into similarly cut pSP64 zCrtc1-flag backbone (PCR-based site-directed mutagenesis approach). This construct is known as pSP RzCrtc1-flag (Appendix 4).

2.1.3. Plasmid used in luciferase assay

pRL Renilla is a DNA plasmid for Renilla Luciferase control reporter (Promega). CRE-luc is a firefly luciferase reporter gene driven by upstream cAMP response element (CRE) and minimal promoter (gift from Walter Born).

Zebrafish crtc1 was PCR amplified with forward primer PR71 (5’-CAACGCTCTGTGTTTTGGAG-3’) and reverse primer PR72 (5’-CCACTGACCAGATGGTGAAC-3’) from cDNA derived from 48 hpf larvae. The amplicon was then cloned into pCR Blunt II TOPO vector. To generate DNA construct expressing Crtc1 with C-term FLAG epitope in pcDNA3.1 expression vector, crtc1 cDNA was Pfu amplified using M13R (5’-CAGGAAACAGCTATGACCATG-3’) and PR69 (5’-AGCTCTAGATATCCAGATCCATGCcGAAAGGT-3’). The amplicon was then cut with HindIII and EcoRV. Linearized product was ligated into similarly cut and dephosphorylated pcDNA3.1 vector (see section 3.1 for detail about early and late crtc1b variant). These constructs are named
pcDNA3.1 early Crtc1b, and pcDNA3.1 late Crtc1b (Appendix 4). In addition, EcoRI-XbaI DNA fragment carrying Crtc1-flag from pcDNA3.1 was sub-cloned into pCS2+ vector and designated as pCS2+ Crtc1-flag (Appendix 4).

2.2. Chemicals, reagents, antibodies and zebrafish

2.2.1. Antibodies

List of antibodies used in this study: Goat polyclonal antibodies (pAb) against C-terminus of human CRTC1 (Santa Cruz, sc-46268), rabbit pAb against the first 42 amino acids at the N-terminus of human CRTC1 (Millipore, ST1098), mouse monoclonal antibody (mAb) against anti-FLAG M2 (Sigma, F1804), mouse mAb against anti-β-Actin (ActB; Sigma, A1978). Secondary antibodies used in Western analysis were mostly conjugated to horseradish peroxidase (HRP).

2.2.2. DNA primers

Table 2.1 List of primer sequence used in semi-quantitative RT-PCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>crtc1</td>
<td>TCCCTCAGAACATCCGTCTC</td>
<td>TTGTGGCTCCCCAGTAAAACC</td>
</tr>
<tr>
<td>crtc1b</td>
<td>CAGCAGTTGGCTCAGTATGC</td>
<td>AGGAGAGAGCTGCTGGTCAT</td>
</tr>
<tr>
<td>actb</td>
<td>CCCAGAGGAGCACCCTGCTCAGAGA</td>
<td>GTGTTGGCATACAGGTCCTACGGATGTCG</td>
</tr>
<tr>
<td>eef1a11l</td>
<td>CGCCCGTGCCAATGTAACCA</td>
<td>TTGCCAGCACCACCGATTTTC</td>
</tr>
</tbody>
</table>

Table 2.2 List of primer sequence used in quantitative RT-PCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>cdkn1a (p21)</td>
<td>CGGAATAAACGGTGTCGTTCT</td>
<td>CGCAAACAGACGAACCTCACT</td>
</tr>
<tr>
<td>actb</td>
<td>TTCCTTCTGGGTATGGAATC</td>
<td>GCACTGTGTTGGGCATACAGG</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>GAAACGGCTACCACATCCAA</td>
<td>CGCTATTGGGAGCTGGAAACC</td>
</tr>
</tbody>
</table>
2.2.3. Mammalian culture media

DMEM high glucose (Invitrogen, cat. no. 12100-046) was used in HEK-293T (Human embryonic kidney cell line or in short 293T) cell culture. Opti-MEM I (Invitrogen, cat. no. 51985-091) was used in transfection with Lipofectamine 2000 (see section 2.9.1.) and electroporation with Bio-Rad electroporator (see section 2.10). Fetal bovine serum (or in short FBS; Hyclone, cat. no. SH30071.03) was heat inactivated at 56ºC for 30 min prior to addition to culture media as growth supplement at final concentration of 10% (v/v).

2.2.4. Zebrafish strain

Wild-type zebrafish stock was procured from Singapore Temasek Life Sciences Laboratory (TLL; http://www.tll.org.sg/) and belongs to the AB genotype/strain (refer to http://zebrafish.org/zirc/fish/lineAll.php). Ethical statement can be referred to in Section 2.13.

2.3. ISH staining of zebrafish embryos

*In situ* hybridization technique provides both the spatial and temporal expression profile of the gene of interest in embryos during development. In this approach, epitope-tagged-RNA probe is hybridized to target RNA in fixed embryos and followed by immunodetection and by colorimetric labeling. In order to take temporal snapshots of gene expression during zebrafish development, embryos were sampled at different developmental stages as described by Kimmel et al. (1995), e.g. 1-cell stage embryos were sampled to represent the zygote period and 16-cells embryos represented the cleavage period etc. (see Table 2.6). ISH was performed according to Thisse and Thisse (2007) and is described in the following subsections.

2.3.1. Antisense labelled RNA probe synthesis

The cDNAs of genes-of-interest were usually cloned into DNA vector containing upstream and downstream phage promoters flanking the cloning site. Prior to the synthesis of labelled RNA, cDNAs in plasmids were linearized with restriction enzymes with specific sites located 5’ (for antisense probes) or 3’ (for antisense probe)
to the inserts and then purified using phenol-chloroform extraction method or PCR purification kit (Qiagen). Synthesis of labelled RNA probes was performed using DIG RNA Labelling Kit (Roche) as according to the manufacturer’s protocol.

2.3.2. Prevention of melanin pigmentation by phenylthiourea (PTU)
Melanin pigmentation in embryos older than 24 hpf could be prevented by treating embryos with 0.0012% (w/v) PTU (Sigma, P7629) in egg water (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄ supplemented with 0.0002% [w/v] methylene blue) at the end of gastrulation. Fresh PTU in egg water was exchanged daily in embryos older than 24 hpf. Alternatively, melanin pigment can be removed by hydrogen peroxide treatment in post-fixed embryos.

2.3.3. Fixing embryos
Embryos were collected according to their developmental stages (see section 2.5) and fixed in 4% (w/v) paraformaldehyde (PFA) in PBS solution overnight at 4°C. Embryos older than 24 hpf were normally dechorionated before fixation. After PFA fixation, embryos were dehydrated in methanol and stored at -20°C, overnight.

2.3.4. ISH and washes
Fixed embryos in methanol were transferred to a 24-well plate. Successive dilution of methanol in PBST (PBS supplemented with 0.1% Tween 20) was performed to rehydrate the embryos by incubating them for 5 min each in 75% (v/v), 50% and 25% methanol. The embryos were then equilibrated in PBST for 5 min. Embryos were then permeabilized in PBST containing 10 μg/ml of Proteinase K at room temperature for the time indicated in Table 2.3 (depending on the developmental stage).
Table 2.3 Duration of 10 µg/ml proteinase K permeabilization in relation to the developmental stage of embryos

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Duration of proteinase K treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \leq 75% ) epiboly</td>
<td>0 to 30 sec</td>
</tr>
<tr>
<td>( \leq 6 ) somites</td>
<td>1 min</td>
</tr>
<tr>
<td>( \leq 18 ) somites</td>
<td>3 min</td>
</tr>
<tr>
<td>Prim-5 to Prim-25 (24 to 36 hpf)</td>
<td>10 min</td>
</tr>
<tr>
<td>( &gt; 48 ) hpf</td>
<td>30 min</td>
</tr>
</tbody>
</table>

Permeabilized embryos were fixed in 4% PFA at room temperature for 20 min. The embryos were then washed in PBST for five times, 5 min for each. At this stage, the embryos were transferred (or allocated) into 1.5 ml tubes. In the prehybridization step, the embryos were incubated in Hyb(plus) buffer (50% formamide, 5x SSC, 0.1% Tween 20, 50 µg/ml heparin and 500 µg/ml baker's yeast tRNA in DEPC treated water; 700 µl Hyb[plus] per 50 embryos) at 65ºC in a waterbath for 2 hrs. The buffer was then discarded, replaced by fresh Hyb(plus) buffer containing 0.15 to 0.5 ng/µl labelled RNA probe and followed by incubation at 65ºC for overnight.

On the next day, Hyb(plus) was discarded and embryos were successively washed with prewarmed 75% (v/v), 50%, and 25% Hyb(plus) in 2x SSCT (2x SSC containing 0.1% Tween 20) at 65ºC, 10 min for each. The embryos were then equilibrated in prewarmed 2x SSC at 65ºC for another 10 min. The 2x SSC was discarded, replaced with 0.2x SSC and then incubated at 70ºC for 30 min. This step was repeated once more. After the SSC washes, the embryos were successively washed in serially diluted 0.2x SSC at 75% (v/v), 50%, and 25% in PBST at room temperature, 5 min for each wash. The embryos were subsequently equilibrated in PBST for another 5 min.

2.3.5. ISH and Immunodetection

After embryos were equilibrated in PBST, the solution was discarded and fresh blocking solution (4 mg/ml sheep serum, 2 mg/ml BSA in PBST) was added. The embryos were incubated in this solution at room temperature for 1 hr. After blocking,
the solution was discarded and fresh blocking solution containing alkaline phosphatase conjugated to Fab fragment of anti-DIG antibody (1:2000 dilution; Roche, cat. no. 11093274910) was added. The embryos were incubated antibody solution at 4°C for overnight.

On the next day, the antibody solution was discarded and PBST was added. The embryos were incubated in PBST at room temperature for 15 min. This step was repeated 5 more times. After the last wash, PBST was discarded and freshly prepared staining buffer (0.1 M Tris-Cl, pH 9.5, 50 mM MgCl₂, 0.1 M NaCl, 0.1% Tween 20) was added. The embryos were incubated in this solution for 5 min. This step was repeated 2 more times. After the embryos were equilibrated in staining buffer, the solution was discarded and replaced with staining buffer containing 337 µg/ml NBT (Roche, cat. no. 11383213001) and 175 µg/ml BCIP (Roche, cat. no. 11383221001). The embryos were incubated in room temperature and protected from light for the colour (or staining) to develop. Colour development was stopped when the desired staining intensity was reached. The staining was stopped by removing the solution and replaced with PBST. The embryos were incubated in the solution for 5 min. This step was repeated 2 more times. After the last wash, the PBST was removed and replaced with 4% PFA. The embryos were post-fixed at room temperature for 2 hrs (or 4°C for overnight). On the next day, the embryos were successively incubated in 25%, 50% and 75% methanol in PBST at room temperature, 5 min for each. After the last wash, the solution was removed and PBST was added. The embryos were incubated at room temperature for 5 min. Next, the PBST was removed and the embryos were mounted in glycerol. Visualization and documentation of stained embryos were performed on Stereomicroscope model SteREO Lumar.V12 (Zeiss; see section 2.10.1).

2.4. Morpholino injection

2.4.1. Calibration of injection volume

Before each injection of morpholino (or any mixture), pulled micropipette was calibrated first. Micropipette was prepared from borosilicate glass capillary (Sutter Instrument cat. no. BF100-58-10) which was pulled by P-2000 laser based micropipette puller (Sutter Instrument) with the following setting: HEAT = 330, FIL =
5, VEL = 10, PUL = 100. A solution containing MO and phenol red indicator dye was back-loaded into a micropipette. The loaded micropipette was fixed onto a micro-manipulator probe connected to the Milli-Pulse Pressure Injector (MPPI)-2 (Applied Scientific Instrumentation, Inc.). The micropipette tip was then broken with a pair of tweezers under a microscope to produce pointed tip. The injector (or microinjector) was set according to manufacturer’s instruction (or ~30 psi). In order to calibrate injection volume, the pulse duration was set to 5 and injection volume was pulse-dispensed onto a micrometer (Fisher, cat. no. 12-561-SM1) overlaid with mineral oil. This was done under the microscope and the diameter (d) of the spherical mixture was registered. The volume was calculated as $4/3\pi r^3$, in which $r$ denotes radius in mm (or $d/2$). The volume in mm$^3$ is equivalent to microliter ($\mu l$) or $10^3$ nanoliter (nl). The following table illustrates the relationship between the diameter (d) versus the corresponding volume (nl) of injection (Table 2.4). The required injection volume was determined by adjusting the pulse duration to produce desired diameter of spherical volume on the micrometer.

<table>
<thead>
<tr>
<th>Injection volume (nl)</th>
<th>Diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.07816</td>
</tr>
<tr>
<td>0.5</td>
<td>0.09847</td>
</tr>
<tr>
<td>0.75</td>
<td>0.11272</td>
</tr>
<tr>
<td>1</td>
<td>0.124065</td>
</tr>
<tr>
<td>1.25</td>
<td>0.133645</td>
</tr>
<tr>
<td>1.5</td>
<td>0.142019</td>
</tr>
<tr>
<td>1.75</td>
<td>0.149507</td>
</tr>
<tr>
<td>2</td>
<td>0.156312</td>
</tr>
<tr>
<td>2.5</td>
<td>0.168382</td>
</tr>
<tr>
<td>3</td>
<td>0.178932</td>
</tr>
</tbody>
</table>

2.4.2. Microinjection technique

Embryos at 1 cell to 8 cells stage were arranged on a 1% agarose with parallel angled trenches/grooves prepared from a mould. Injection was done near to the embryo proper or at the centre of the yolk (in order to maximize MO intake by cytoplasmic streaming of the yolk).
2.4.3. Morpholino design and order

Morpholinos were designed by Gene-Tools (http://www.gene-tools.com/) based on either the 5’ untranslated sequence (5’-UTR) near translation start site or intron-exon junctions sequences of genes-of-interest (GOI) submitted to them. Instruction for submission of the DNA sequence of GOI is available on their website. Morpholino that targets human *beta-globulin* intron mutation was used as a control MO (Vivo-MO standard control oligo, Gene-Tools). This standard MO has 32% GC content. The following table lists translation and splice morpholinos with their corresponding sequences used in this study.

Table 2.5  List of translation and splice morpholinos and their corresponding sequences

<table>
<thead>
<tr>
<th>Morpholinos</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Translation blockers</strong></td>
<td></td>
</tr>
<tr>
<td><em>crtc1</em> tMO1</td>
<td>CGGATTGGTTTGAGGACGCCATCTCTC</td>
</tr>
<tr>
<td><em>crtc1</em> tMO2</td>
<td>GTTCTTCTCCAAACACAGAGCGTT</td>
</tr>
<tr>
<td><em>crtc1b</em> tMO1</td>
<td>GGATTATCGAGGTCGCATGTTC</td>
</tr>
<tr>
<td><em>crtc1b</em> tMO2</td>
<td>TCCTCTTGGGCTCCGCTCTGTTCA</td>
</tr>
<tr>
<td><em>p53</em> tMO</td>
<td>GACCTCCTCTCCACTAAACTACGAT</td>
</tr>
<tr>
<td><strong>Splice blockers</strong></td>
<td></td>
</tr>
<tr>
<td><em>crtc1</em> splMO1</td>
<td>AGCACTGATGAAACACAGATATGGA</td>
</tr>
<tr>
<td><em>crtc1</em> splMO2</td>
<td>TTTCTTCTACGGGAGGGGAATAT</td>
</tr>
<tr>
<td><em>crtc1b</em> splMO3</td>
<td>ACGAGTGCTGAAACAAGACATATT</td>
</tr>
<tr>
<td><em>crtc1b</em> splMO4</td>
<td>GCTGAAACGAGTGCAAAACAAGCTGCGA</td>
</tr>
<tr>
<td>Vivo-MO standard control</td>
<td>CCTCTTTACCTCATCTTACATTATA</td>
</tr>
</tbody>
</table>

2.4.4. Morpholino injection and rescue

For rescue experiments, capped RNA was synthesized using MAXIscript SP6 Transcription Kit (Invitrogen, cat. no. AM1308). The synthesized RNA was purified as according to the kit’s instruction. To increase the stability of the capped transcript, cDNAs were cloned into pSP64 vector containing polyA tail. Capped and polyA tailed transcript derived from pSP64 RzCrtc1-flag was used for rescuing phenotype elicited
by knocking down endogenous *crtc1* expression by translation morpholino (*crtc1* tMO1). As mentioned, transcript encoding RzCrtc1 is tMO1-resistant (see section 2.1.2.). Embryos at 1 to 4-cells stages were co-injected with both morpholino and synthesized RNA. Co-injection of both the morpholino and the RNA produced minimal physical damage caused by repeated injection.

### 2.5. Staging of the zebrafish embryos

Staging of the zebrafish embryos (or larvae) was done according to Kimmel et al. (1995) by referring to observable landmarks on the embryos (e.g. otic vesicle length, OVL; yolk extension and diameter ratio; pectoral fin morphology, e.g. high fin vs. long fin; epiboly etc.). For *in situ* hybridization experiment to determine spatiotemporal expression of *crtc1* (or *crtc1b*), embryos were sampled to represent each developmental period (see Table 2.6).

<table>
<thead>
<tr>
<th>Developmental period</th>
<th>Developmental stages</th>
<th>Hours post-fertilization (hpf)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zygote</td>
<td>1-cell</td>
<td>0</td>
</tr>
<tr>
<td>Cleavage</td>
<td>2-cell to 64-cells</td>
<td>0.75 to 2</td>
</tr>
<tr>
<td>Blastula</td>
<td>128-cells to 30%-epiboly</td>
<td>2.25 to 4.67</td>
</tr>
<tr>
<td>Gastrula</td>
<td>50%-epiboly to bud</td>
<td>5.25 to 10</td>
</tr>
<tr>
<td>Segmentation</td>
<td>1-somite to 26-somites</td>
<td>10.33 to 22</td>
</tr>
<tr>
<td>Pharyngula</td>
<td>Prim-5 to high-pec</td>
<td>24 to 42</td>
</tr>
<tr>
<td>Hatchling</td>
<td>Long-pec to protruding-mouth</td>
<td>48 to 72</td>
</tr>
</tbody>
</table>

### 2.6. Immunodetection

#### 2.6.1. Lysate preparation

Lysate was prepared from cultured cells by lysis in RIPA buffer (50 mM Tris-Cl, pH 7.4, 0.15 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (Roche cat. no. 11697498001). The lysate was incubated on ice, 10 min and cleared by centrifugation at maximum speed for 10 min.
To prepare lysate from zebrafish embryos with minimal yolk contamination, the method was done according to Waskiewicz et al. (2001). Briefly, 20 dechorionated embryos were placed into 1.5 ml tube and 167 µl of homogenization buffer (20 mM HEPES, pH 7.5, 10 mM EGTA, 2.5 mM MgCl₂) supplemented with protease inhibitors was added to it. The embryos were homogenized with pellet pestle grinder (Sigma, cat. no. Z359971). The homogenate was then centrifuged at 16,000 g for 5 min at 4°C. The supernatant containing yolk content was discarded. The resultant cell pellet was resuspended in 1 x Laemmli buffer. The lysate was sonicated to reduce viscosity and then boiled for 5 min before proceeding to Western analysis (see section 2.6.3).

2.6.2. Immunoblot and immunodetection

Immunoblot and immunodetection was performed according to standard procedure (Maniatis et al., 1982). Normally, lysates were mixed with 1/3 volume of 4 x Laemmli buffer (0.25 M Tris-Cl, pH 6.8, 8 % SDS, 40 % glycerol, 0.04 % bromophenol blue and 20 % 2-mercaptoethanol) and heated at 65°C for 15 min (or boiled for 5 min). The lysates were then resolved in SDS-PAGE and electroblotted into polyvinylidene fluoride (or PVDF) membrane (Thermo Scientific, cat. no. 88518). Membrane blocking was performed using 5% low-fat milk (Anlene; or blotting grade blocker from Bio-Rad, cat. no. 170-6404) in TBST (0.05% Tween 20). Antibodies used for detection is listed in section 2.2.1. HRP activity in the enzyme conjugated secondary antibodies were detected using HRP substrates such as Luminata Crescendo Western HRP substrate (Millipore, cat. no. WBLUR0500) or WesternBright ECL (Advansta, cat. no. K-12045-D50). The luminescent signal was captured by Medical X-ray film (Kodak, cat. no. 6550016) and developed using Kodak X-OMAT 2000 machine.

2.7. Real time quantitative polymerase chain reaction (PCR) amplification and detection

2.7.1. Total RNA isolation

Total RNA from cell culture or zebrafish embryos was isolated using Trizol reagent (Invitrogen cat. no. 15596-026) as according to the manufacturer’s instruction. In
order to homogenize zebrafish embryos in Trizol reagents, two mechanical means were used (and worked equally well), i.e. homogenization through a 26 ½ G syringe or by pellet pestle grinder.

2.7.2. First strand cDNA synthesis
Prior to first strand cDNA synthesis, the total RNA was treated with RNase-free DNaseI (Thermo Scientific cat. no. EN0521) to remove genomic DNA. First strand cDNA synthesis was performed using SuperScript III First-Strand Synthesis System (Invitrogen cat. no. 18080051) as according to the manufacturer’s instruction. As a control, RT minus (without reverse transcriptase enzyme) was used to test the presence of genomic DNA contamination during RT-PCR.

2.7.3. RT-qPCR primer design
For qPCR, primer pairs were designed according to standard procedure (with emphasis on homo- or hetero-dimerization or hair-pin structure prediction). In addition, primer pairs were positioned in different exons (flanking an intron) in order to minimize the probability of quantifying the amplicon derived from genomic DNA.

2.7.4. Real time reverse transcriptase-qPCR (RT-PCR) using SYBR Green
qPCR reaction was performed using SYBR Green dye (Invitrogen cat. no. 4472908) and detected on a Bio-Rad iQ-5 Optical System platform as according to manufacturers’ instructions. The result was analysed using Bio-Rad iQ5 bundled software.

2.8. Semi-quantitative RT-PCR
Semi-quantitative RT-PCR was performed using Taq DNA polymerase (Thermo Scientific cat. no. EP0401) as according to manufacturer’s instruction. The PCR reaction was completed before the depletion of PCR components (or saturation of PCR amplification). This was determined by serial removal of amplification product at 10,
15 and 30 PCR cycles for agarose gel resolution by electrophoresis and detection. The use of semi-qPCR was to provide qualitative assessment of transcript abundance.

2.9. Luciferase reporter assay

2.9.1. Transfection of reporter and experimental DNA plasmids in cell culture
Reporter DNA plasmids (or constructs) and experimental constructs were prepared in a master-mix (wherever possible) and aliquotted for each experimental parameter. The DNA mixture was then transfected into cultured cells with Lipofectamine 2000 (Invitrogen cat. no. 11668019) as according to manufacturer’s instruction. Transfectants were harvested >12 hours later.

2.9.2. Luciferase reporter assay and detection
Luciferase reporter assay was done using a Dual-Luciferase reporter assay system (Promega cat. no. E1960) as according to the manufacturer’s instruction. The transfectants were lysed with bundled Passive Lysis buffer (Promega) and assayed/detected using Thermo Scientific Fluoroskan Ascent FL luminometer with bundled software.

2.10. Electroporation
Electroporation of 293T cells with a combination of crtc1 tMO1 and DNA plasmid expressing either Crtc1-flag or MO-resistant Crtc1-flag was performed using Gene Pulser Xcell electroporation system (Bio-Rad) as according to manufacturer’s setting for 293T (accessible on the machine, i.e. preset program for 293T with square wave pulse at 110 V for 25 msec). Briefly, 10 µg of plasmid DNA and 1 µg of MO were added into 293T cells in Opti-MEM (or growth media without serum) at 3 x 10⁶ cells/ml and final volume 400 µl. The content was transferred into a 4-mm-gap electroporation cuvette (Peqlab; cat. no. 71-2030) and kept on ice before and after electroporation. After electroporation, ~0.5 ml of media (DMEM supplemented with 10% FBS) 1 was added to the cells. The cells were transferred into 6-well plate using
sterile Pasteur pipette and incubated at 37°C, 5% CO₂ for 24 (or up to 48 hours; 293T has average doubling time of 20 to 24 hpf in our hand).

2.11. Microscopy and imaging analysis

Stereomicroscope model SteREO Lumar.V12 (Zeiss) was used for image processing and documentation. This was done using the bundled AxioVision software.

ImageJ v1.47t was used to analyse ISH images. ImageJ is a freely available image analysis/processing software (http://rsbweb.nih.gov/ij/). Image processing and analysis was done according to user manual provided.

2.12. Statistical analysis

All statistical analyses to determine the significant difference of two samples/measurements were performed using the two-tailed unpaired t-test. The definition for N (or number of measurements/observations) is described in each figure/table caption.

2.13. Zebrafish handling and care

All animal work was in concordance to the Institutional Animal Care and Use Committee (IACUC) guidelines at Nanyang Technological University (IACUC number: ARF SBS/NIE-A 0144 AZ). Animal handlers were required to attend training workshops on responsible care and use of laboratory animals (RCULA).
CHAPTER 3 : RESULTS

3.1. Spatial and temporal expression of zebrafish \textit{crtc1} and \textit{crtc1b} during early embryonic development

In order to determine zebrafish (\textit{D. rerio}) \textit{crtc1} (Gene Accession no. xm_003199425) and \textit{crtc1b} (Gene Accession no. nm_001077457) spatiotemporal expression, I employed both the \textit{in situ} hybridization method and semi-quantitative RT-PCR to assess these transcripts during embryonic development. By \textit{in situ} hybridization, both \textit{crtc1} and \textit{crtc1b} were detected at 1-cell stage embryos when zygotic expression was absent (Fig. 3.1 and Fig. 3.2). This indicated that \textit{crtc1} and \textit{crtc1b} RNAs are maternally deposited during oogenesis. In subsequent embryonic stages from the cleavage period to the segmentation period, both \textit{crtc1} and \textit{crtc1b} displayed similar zygotic expression patterns in which both were expressed ubiquitously and were not spatially restricted to any tissue or domain. At prim-5 stage (24 hpf), \textit{crtc1} and \textit{crtc1b} were detected in the eye, brain, and the mesoderm derivatives (e.g. somites). However, at long-pec stage (48 hpf), \textit{crtc1} and \textit{crtc1b} expression in the somites decreased while expression in the brain remained high. A distinguishing aspect of \textit{crtc1} and \textit{crtc1b} expression at 48 hpf is that \textit{crtc1} RNA was detected at the caudal fin fold whereas it is not for \textit{crtc1b} (compare long-pec stage of Fig. 3.1 and Fig. 3.2).

Semi-quantitative RT-PCR was used to validate observations from \textit{in situ} hybridization. Similar to \textit{in situ} hybridization results, maternal transcripts for both \textit{crtc1} and \textit{crtc1b} were detected at 1-cell stage. Both \textit{crtc1} and \textit{crtc1b} expression persisted up to sphere stage (or the blastula period). At germ-ring stage (or the gastrula period), both transcripts showed decreased expression. The change in expression level could be due to the mid-blastula transition (MBT), which is a stage when zygotic genes are activated followed by the turn-over of maternal RNAs (Marlow, 2010). The reduced \textit{crtc1} and \textit{crtc1b} transcript levels persisted until long-pec stage (48 hpf in the hatchling period) and then started to climb afterwards (Fig. 3.3C).

By using semi-quantitative RT-PCR, an unexpected higher molecular weight (MW) \textit{crtc1b} transcript variant was detected at 24 hpf and in subsequent developmental stages (Fig. 3.3C). This variant was absent in the maternal RNA and in
earlier developmental stages. In order to identify this higher MW PCR product, direct sequencing of the gel isolated PCR product was performed (Khorana et al., 1994). Sequencing results showed that the higher MW DNA possessed an exon denoted as exon 11 in relation to crtc1b exon 1 (Fig. 3.3B) that was retained in the “late” transcript variant, whereas this exon was selectively spliced out in maternal RNA (or “early” transcript variant). In comparison, the equivalent exon in the crtc1 gene was not selectively spliced-out from the maternal RNA. Instead, this exon in crtc1 was included in maternal and zygotic expression (Fig. 3.3). The significance of regulating the alternative splicing of crtc1b exon 11 during embryonic development is currently not known.

Semi-quantitative RT-PCR of housekeeping genes beta actin (actb, Gene Accession no. nm_181601.4) and eukaryotic translation elongation factor (eef1a1l1, Gene Accession no. nm_131263.1) showed that these genes were expressed at low levels during early stages of embryonic development and their expression increased after initiation of zygotic gene expression at the germ-ring stage (Fig. 3.3C). Fifteen PCR cycles were chosen for qRT-PCR to avoid saturating the signal. The dynamic expression of these “housekeeping” genes during embryonic development has been reported elsewhere suggesting that choosing reference genes for quantitative PCR to normalize gene expression during developmental stages is a challenge (McCurley and Callard, 2008; Tang et al., 2007).

### 3.2. Characterization of zebrafish Crtc1 and Crtc1b

The predicted translation products of crtc1 and crtc1b have a very high sequence similarity (63% protein identity) as compared to other Crtcs in zebrafish. Phylogenetic analysis showed that both Crtc1 and Crtc1b are clustered together within the “CRTC1” clade when they are aligned with zebrafish Crtc2, Crtc3 and CRTC1s from other organisms (Fig. 3.4). This suggests that Crtc1 and Crtc1b might belong to the same CRTC1 “subfamily” of CRTC1s. The presence of duplicated homologous crtc1 genes (e.g. crtc1 and crtc1b) is unique to teleost and is absent in mammals.
crtc1 spatio-temporal expression during early development was examined by in situ hybridization. Zebrafish embryo developmental stages are according to Kimmel et al. (1995).

(A-I) The developmental stage of each labeled embryo is described at the bottom of the image. Red scale bar = 100 µm. Refer to Appendix 5a for sense control probe staining.
Figure 3.2 Spatio-temporal expression of *crtclb* during early zebrafish development

*crtclb* spatio-temporal expression during early development as demonstrated by *in situ* hybridization. (A-G) The developmental stage of each labeled embryo is described at the bottom of the image. Red scale bar = 100 µm. Refer to Appendix 5b for sense control probe staining.
Figure 3.3 Semi-quantitative PCR assessment of *crtc1* and *crtc1b* transcript expression level during early zebrafish development

(A-B) Dark grey boxes represent exons while lines denote introns. Scale bars under exon and intron denote 100 bp and 1000 bp, respectively. (A) The positions of PCR primers on the *crtc1* gene locus are shown as opposing arrows. (B) Similarly, the positions of *crtc1b* primers on its gene locus are shown. (C) Position of protein domains of Crtc1 and Crtc1b late isoform are shown. N-term = N-terminal domain that interacts with bZIP domain of Creb protein, NLS = nuclear localization sequence, NES = nuclear export sequence, and TAD = transactivation domain. (D) Semi-quantitative RT-PCR was performed using cDNA derived from embryos at different developmental stages encompassing 1-cell to 4 dpf stages. Attempt to normalize using housekeeping genes *beta actin (actb)* and *eukaryotic translation elongation factor (eef1a1l1)* revealed the highly dynamic expression of these housekeeping genes, hence corroborating previous works (McCurley and Callard, 2008; Tang et al., 2007). NTC = no template control. The result shown is a representative of two independent experiments.
Based on RT-PCR and sequencing results, *crtc1b* expression was different from *crtc1* in term of exon usage during maternal and zygotic transcription. *crtc1b* exon 11 was shown to be selectively spliced during maternal and zygotic transcription (see section 3.1). To determine if amino acid sequence derived from exon 11 is conserved in *crtc1* subfamily, multiple sequence alignment of zebrafish Crtc1 and Crtc1b with orthologous CRTC1 members of other organisms was performed. The alignment showed that the amino acid sequence derived from exon 11 of *crtc1b* is highly conserved in other species (Appendix 1). Next, in order to determine if amino acid sequence from exon 11 is conserved in other CRTCs family members as well, multiple sequence alignment of CRTCs family members (e.g. CRTC1, CRTC2 and CRTC3) was performed. Even though inclusion of sequences from other CRTCs family members reduced the conservation quality of this region (Appendix 2), it does not preclude the possibility that the protein domain encoded by this exonic region could play a unique function in CRTC1 orthologs while not in other CRTCs (e.g. CRTC2 and CRTC3) family members.

In order to determine if both Crtc1 and Crtc1b possess transcription coactivator activity as do their counterparts in other organisms, a CRE-luciferase reporter assay
was used for the test. The reporter plasmid is composed of a cAMP response element (CRE) upstream of a minimal promoter driving a luciferase gene (gift from Walter Born). Both Crtc1 and Crtc1b were able to potentiate endogenous CREB activity when cotransfected with the reporter plasmid in HEK293T cells (Fig. 3.5B and 3.6B) suggesting that both Crtc1 and Crtc1b are functional CREB coactivators. Also, Crtc1 showed higher activation compared to Crtc1b (compare Fig 3.5B and 3.6B). In addition, early and late isoforms of Crtc1b showed similar enhancement of Creb transcriptional activity in the luciferase assay (Fig. 3.5B) suggesting that the inclusion of exon 11 in the late isoform did not alter the coactivator activity of Crtc1b under these conditions.

All the plasmid constructs expressing Crtc1, Crtc1b early or late isoforms used in the luciferase reporter assays were transfected in HEK293T and detected by Western analysis (Fig. 3.5A and 3.6A). A discrepancy between the observed and predicted molecular masses of Crtc1b early and late isoforms was noted. However, for the early isoform to migrate slower than the late isoform (with an additional exon 11) was surprising considering that the early isoform was smaller in size than the late isoform.

3.3. **Colocalization of crtc1 (and crtc1b) transcript expression with meis1 (nm_131893) in 24 hpf zebrafish embryo**

The cooperative action of MEIS1A/MEIS1B and CRTCs to activate HOX/PBX/MEIS1 target genes (e.g. murine Hoxb2 r4 enhancer and Meis1 promoter) suggests that this collaboration can be expanded to other untested MEIS1 target genes involved in embryonic development and other biological processes such as hematopoiesis or cancer (Goh et al., 2009). In order to determine the developmental processes involving the convergence of MEIS1 and CRTCs (or specifically CRTC1) activity in programming such processes, I relied on comparing the spatial expression of crtc1 (or crtc1b) and meis1 (formerly known as meis1.1) transcripts in zebrafish embryos at specific developmental stages. The rationale is that crtc1 (or crtc1b) will cooperate with meis1 in tissues where genes are co-expressed.
Figure 3.5  Crtc1b coactivator activity in CRE-luciferase reporter assays.

(A) Western detection of Crtc1b early and late isoforms expressed in transfected HEK293T cells. (B) CRE-luciferase reporter is composed of a cAMP response element (CRE) upstream of minimal promoter driving the transcription of luciferase reporter gene. The reporter plasmid was cotransfected with pcDNA3.1 expressing either Crtc1b early or late isoform in HEK293T cells. Crtc1b early and late isoforms were able to potentiate endogenous CREB activity in CRE-luciferase reporter. The results were derived from two independent experiments.

Figure 3.6  Crtc1 coactivator activity in CRE-luciferase reporter assay

(A) Western detection of Crtc1 transfected in HEK293T cells. (B) CRE-luciferase reporter plasmid was cotransfected with either empty vector or pcDNA3.1 expressing Crtc1 in HEK293T cells. Crtc1 was able to potentiate endogenous CREB activity in CRE-luciferase reporter. The results were derived from two independent experiments.
Spatiotemporal expression of zebrafish *meis1* at selected embryonic stages was determined using *in situ* hybridization. As a complement, *meis1* expression data can also be accessed in The Zebrafish Model Organism Database (ZFIN) depository ([http://zfin.org/](http://zfin.org/)). *meis1* transcript is detected as early as 50% epiboly and its expression is spatially restricted to the neural plate and presumptive segmental plate (ZFIN). At the segmentation period, *meis1* expression was detected in the optic primordium, midbrain, hindbrain, neural tube and segmental plate (Fig. 3.7A). The segmental plate (or presomitic mesoderm) will give rise to the transient somites and subsequently all other mesodermal derivatives such as cartilage, bone, tendon, muscle, dermis, and endothelial cells along the anterior-posterior axis of the embryo. At prim-5 (24 hpf), *meis1* transcript expression remained unchanged in the eye field, midbrain, hindbrain, neural tube and somites. In addition, *meis1* expression was detected in the olfactory bulb and otic vesicle. At long-pec stage (48 hpf), *meis1* expression could still be detected in the eye, midbrain tectum, hindbrain and neural tube but showed marked reduction in the somite derivatives (e.g. body muscle). On top of this expression pattern, *meis1* was also found in the branchial arches and pronephric duct (Fig. 3.7C).

Murine *Meis1* expression is detected as early as E3.5 (Theiler stage, TS4) by qRT-PCR (Guo et al., 2010). At E7.0 (TS10), *Meis1* is detected in the heart, brain and lung tissues (Nakamura et al., 1996). This corresponds to the pre-segmentation period, whereas embryonic segmentation starts around E7.5 onwards. *In situ* hybridization shows that *Meis1* is present at E7.75 (TS11) in embryonic mesoderm, while at E8.5 (TS13) *Meis1* is detected in neural tube and mesoderm. In subsequent developmental stages, *Meis1* is detected in the brain, eye, olfactory lobe, ear, spinal cord and trunk mesoderm ([http://www.emouseatlas.org/emage/home.php](http://www.emouseatlas.org/emage/home.php)) showing similar expression pattern as zebrafish. Similar expression pattern was also seen in chick at HH stage 14 (i.e. during segmentation period) in which *Meis1* is expressed in the brain, spinal cord, eye, nasal placode, trunk mesenchyme and mesoderm, among others ([http://geisha.arizona.edu; Sánchez-Guardado et al., 2011](http://geisha.arizona.edu; Sánchez-Guardado et al., 2011)).

The overlap of *crtc1/crtc1b* and *meis1* expression domains was assessed across three developmental stages. In the early segmentation period (from 6-somites [12 hpf] to 14-somites stage [16 hpf]), *crtc1* and *crtc1b* was ubiquitously expressed and seemed to overlap with *meis1* expression domains (compare Fig 3.1G or 3.2E with 3.7A). The
overlapping domain of \textit{crtc1} and \textit{crtc1b} with \textit{meis1} expression was more discernible at prim-5 stage (24 hpf), in which they were found in the forebrain, olfactory bulb, eye, midbrain, hindbrain, and in the somites (compare fig 3.1H or 3.2F with 3.7B). Similarly, both transcripts were found in the brain region and eye at the long-pec stage (48 hpf). However, the overlap was not found in the neural tube and body segment (compare fig. 3.1I, 3.2G with 3.7C). Overlapping regions of \textit{crtc1} and \textit{meis1} expression are depicted as green highlight (Fig. 3.8).

Figure 3.7 \hspace{1em} \textbf{Spatiotemporal expression of } \textit{meis1} \hspace{1em} \textbf{during zebrafish development}

(A) \textit{meis1} expression is specific to eye primordium, midbrain, hindbrain, neural tube and segmental plate at 14 somites. (B) \textit{meis1} expression in prim-5(24 hpf) embryos viewed as wholenumount (left panel) and flatmount (right panel). \textit{meis1} is expressed in the eye, olfactory bulb, midbrain, hindbrain, otic vesicle, neural tube, ventral mesoderm and somites. (C) At long-pec (48 hpf), \textit{meis1} is detected in the eye, olfactory bulb, midbrain, hindbrain, spinal cord, branchial arches and pronephric duct. HB = hindbrain, OB = olfactory bulb, OV = otic vesicle, SP = segmental plate. Scale bar = 100 µm.
Figure 3.8  Overlap region of *crtc1* and *crtc1b* with *meis1* expression deduced from *in situ* hybridization staining

Highlighted green is the deduced overlap domain of *crtc1* or *crtc1b* expression with *meis1*. Embryo in top panel is at Prim-5 stage (24 hpf) and embryo at the bottom is at long-pec stage (48 hpf). (1) Forebrain (or olfactory bulb), (2) eye, (3) midbrain, (4) hindbrain, and (5) mesoderm derivatives (e.g. somites). Schematic representations of embryos were taken from Kimmel et al. (1995). Scale bar = 250 μm.

In order to observe better the *in situ* hybridization staining of *crtc1* and *meis1*, the stained embryos were each dissected to remove the bulky yolk and then mounted onto a glass slide and flattened with a coverslip. The flat-mount was then visualized dorsally (Fig. 3.9A). Similar to the above observation, *crtc1* and *meis1* transcripts were found colocalized in the brain (especially midbrain), eye and somites at prim-5 (24 hpf) stage (Fig. 3.9A). This overlap was also found in the hindbrain, but weakly evident in the spinal cord (Fig. 3.9A). To examine the overlapping domain of *crtc1* and *meis1* expression in another perspective, the hybridization signal of flat-mounted embryos was digitally pseudocoloured using ImageJ software. The *crtc1* signal was assigned a red colour and that of *meis1* a green colour. These images were later
merged with the same software and the coincident signal from *meis1* and *crtc1* expression was displayed as yellow (Fig. 3.9B). This confirmed overlapping expression in the olfactory bulb, the laminating layer of the retina, the midbrain and hindbrain.

**Figure 3.9**  Colocation of *crtc1* and *meis1* transcript expression domains in 24 hpf zebrafish embryo

(A) A typical *in situ* hybridization staining of *crtc1* (top panel) and *meis1* (bottom panel) of flat-mounted 24 hpf embryo. (B) *In situ* hybridization stains are converted to pseudo-colours (top and middle panels). These pseudo-coloured signals were then merged (bottom panel) and *crtc1* signal was assigned “red” *meis1* “green”. Co-incident signal are revealed as yellow. White scale bars =100 µm.
3.4. Morpholino mediated knockdown of \textit{crtc1} function

The primary objective of the experiment is to knockdown both \textit{meis1} and \textit{crtc1} (or \textit{crtc1b}) and to identify the embryonic developmental domain(s) that is/are susceptible to interrupt Meis1 and Crtc1 functions. This is considering that MEIS1 and CRTC\textsubscript{S} have been demonstrated previously to cooperate in the transcription of HOX/PBX/MEIS1 target genes. In zebrafish, \textit{meis1} knockdown has been reported (Bessa et al., 2008; Erickson et al., 2010), whereas \textit{crtc1} knockdown has not. To characterize \textit{crtc1} knockdown is therefore important to facilitate interpretation of co-knockdown of \textit{meis1} and \textit{crtc1}.

In zebrafish, there are two \textit{crtc1} members, i.e. \textit{crtc1} and \textit{crtc1b}. These genes displayed similar spatiotemporal expression during embryonic development (see section 3.1). In order to study the developmental role of this gene family, I decided to knock-down either \textit{crtc1} or \textit{crtc1b} with antisense MO oligonucleotide and to characterize each morphant phenotype. I used both types of MOs, i.e. splice or translation blockers, for comparison and to confirm specificity. To distinguish the embryos injected with either splice blocker or translation blocker, injected embryos will be designated as splice morphant or translation morphant where applicable in the following text.

3.4.1. Splice blockers targeting \textit{crtc1} and \textit{crtc1b} disrupt splicing and have pleiotropic effects on zebrafish development

Splice MOs against \textit{crtc1} (or \textit{crtc1b}) were designed to target a 3’ splice acceptor site so as to give rise to a predicted deletion of the C-terminal transactivation domain of Crtc1 and Crtc1b (Fig. 3.10A and 3.11A). The deletion was predicted to incapacitate the corresponding protein’s function as a transcriptional coactivator. To test for the presence of altered splicing product, RT-PCR was used to amplify the exon-intron junctions where MO binding sites were situated (Fig. 3.10A and 3.11A). Altered splicing product was expected to either change the amplicon size as compared to control or render the altered transcript undetectable by RT-PCR. The latter could be due to the use of cryptic splice acceptor sites producing an unstable RNA or aberrant
splicing that removed the PCR primer binding sites from the final product. The PCR binding sites are denoted as P1 and P5 (Fig. 3.10A and 3.11A). I determined that injection of 6 ng of morpholino per embryo was sufficient to produce observable phenotypic changes, and used this amount for subsequent studies. When co-injecting embryos with two splice blocking MOs simultaneously, I used 3ng each for a total of 6ng. This decision was based on the assumption that such amounts would be sufficient to provoke a phenotype.

In embryos which were co-injected with two splice blockers against *crtc1* (e.g. splMO1 and splMO2 at 3 ng each for a total of 6ng), there were two altered splice-variants detected by RT-PCR, i.e. a slow migrating PCR band and a fast migrating band (this altered splicing product is difficult to discern from the normal agarose gel electrophoresis; alternatively Metaphor gel can used for better resolution) when compared to control (Fig. 3.10B). These bands were gel-purified and submitted for direct DNA sequencing. Sequencing results showed that the slower migrating band (denoted as variant 1) incorporated an intron situated immediately after the exon containing splMO1 binding site. Inclusion of the intron sequence was predicted to cause protein truncation due to premature stop codon. In comparison, the fast migrating band (denoted as variant 2) was shown to splice to a cryptic acceptor site downstream of the *bona fide* acceptor site and this was expected to cause a deletion followed by a frame shift mutation and premature stop (Fig. 3.10C). Both the predicted truncated polypeptides lacked the transactivation domain (TAD) and the nuclear export signal (NES). These mutant proteins were similar to the dominant negative CRTC1 as described by Kovac et al. (2007). Single injection of splMO1 did not produce detectable altered splicing product (data not shown), whereas splMO2 injected morphants produced altered splicing product similar to the co-injection of splMO1 and splMO2 (data not shown).

In embryos injected with 6ng of *crtc1b* splice MO 3 (splMO3), there was a fast migrating PCR band detected by RT-PCR in the morphant but absent in control (Fig. 3.11B). This DNA band was purified and submitted for DNA sequencing. Sequencing results suggested a skipping of the exon where the splMO3 binding site was situated (asterisk in Fig. 3.11B). However, the resultant transcript was still predicted to produce an in-frame polypeptide but lacking the 30 amino acids derived from the
skipped exon (Fig 3.11C). In contrast, there was no amplicon detected in embryos injected with the crtc1b splice MO 4 (splMO4) as compared to controls (Fig. 3.11B). A similar result was obtained from co-injection of both splMO3 and splMO4 at 3ng each (total 6 ng). The inability to detect altered splice products by RT-PCR in the latter group could be because the altered transcripts were unstable or lacked sites complementary to the PCR primers. The latter could happen if the alternative splicing was at a cryptic acceptor site situated in the intronic region. Another possibility would be that the splicing happened at a cryptic acceptor site downstream of the primer binding site (middle row of Fig. 3.11B). In either case, the splicing to cryptic acceptor site would remove the PCR primer binding site and hence prevent any amplification.

![Figure 3.10 Splice morpholinos caused altered splicing of crtc1](image)

(A) Position of splMO1 and splMO2 binding sites in the crtc1 gene locus (represented as cyan columns, also in C) and the flanking crtc1 P1 and P5 PCR primers (represented by filled arrows) are shown. Exonic regions coloured blue and indigo are targeted by splMO1 and splMO2 respectively. Exonic regions coloured pink encode the crtc1 TAD region. Light grey regions flanked exons targeted by morpholinos. (B) RT-PCR results showed altered splicing product in 24 hpf morphants co-injected with both splMO1 and splMO2 (at 3 ng each), as assessed by the presence of amplicons of altered size (arrowheads in upper panel). This was in comparison to uninjected embryos as control. Lower panel shows RT-PCR of housekeeping actb gene. (C) Sequencing result suggested that the two altered splicing product (denoted as variants var 1 and var 2) detected in morphants were expected to produce truncated proteins lacking TAD. The expected size of the altered Crtc1 produced from var (1) and var (2) are 231...
aa and 214 aa respectively, out of the full length of 583 aa. Red asterisk denotes premature stop codon. Black column in var 1 represents amino acid sequence derived from the intronic region. PCR amplification with P1 and P5 primers produces 475 bp amplicon for wild type control, 600 bp for var 1, and 452 bp for var 2 in morphants.

**Figure 3.11  Splice MOs caused altered splicing of crtc1b**

(A) Position of splMO3 and splMO4 (represented as cyan columns, also in B) and the flanking crtc1b P1 and P5 PCR primers (represented by filled arrows) are shown. Exon highlighted in green represents exonic region selectively spliced into the late crtc1 variant. However, it was not detected (due to low abundance; see Fig. 3.3C) by RT-PCR in 24 hpf embryos. (B) From upper to bottom row, morphants were injected with either 6 ng of splMO3, 6 ng of splMO4 or both (at 3 ng each for a total of 6 ng). splMO3 injected embryos produced an altered splice product (denoted with an asterisk), whereas splMO4-injected and splMO3+splMO4-injected embryos produced possible unstable altered spliced products not detectable by RT-PCR. (C) Sequencing result suggested that the altered splice product in splMO3 morphants skipped an exon but still produced an in-frame polypeptide with “deletion mutation” in reference to the skipped exon. PCR with P1 and P5 primers is expected to produce amplicon size of 376 bp for wild type control and 289 bp for splMO3 altered transcript in morphants.
In order to characterize the phenotype of *crtc1* and *crtc1b* morphants, a dose-response experiment at 4 ng and 6 ng (or 8 ng in *crtc1b* morphants) was carried out. The dose-response was performed to determine the MO amount per embryo that would elicit reproducible phenotypic changes in >50% of injected embryos without causing toxicity. Toxicity would be revealed by a high rate of lethality and embryos with the so-called “monster” phenotype (Bedell et al., 2011; Bill et al., 2009).

Injection of *crtc1* splice blocker splMO2 at 4 ng and 6 ng per embryo was enough to produce embryos with an abnormal phenotype. The morphant phenotype was more penetrant with increasing dosage such that >50% embryos injected with 6 ng of splMO2 displayed an abnormal phenotype (by gross observation). Embryos at 48 hpf with the morphant phenotype exhibited shortening of body length, abnormal body curvature, retention of fluid in the pericardial cavity and brain, abnormal brain and eye size, and defective notochord with kinks (Fig. 3.12B and 3.12C). The heart was pumping in most morphants but there were no observable red blood cells in circulation in some embryos with severe phenotype (data not shown). In addition, 24 hpf embryos injected with 6 ng of MO showed a higher incidence of probable cellular death in the brain region as distinguished by an opaque area in the brain as reported by Robu et al. (2007) and points to possible non-specific toxicity of this splice blocker. An alternative would be that the aberrantly spliced RNA encoding Crtc1 protein was toxic to the embryo itself. Concurrent with observations by Robu et al. (2007), the opaque region diminished and was no longer apparent after 48 hpf. In comparison, injection of *crtc1* splMO1 at 2 ng did not elicit any phenotype whereas injection at 4 ng and 6 ng produced similar phenotype as splMO2 morphants (data not shown).

Injection of *crtc1b* splice blocker splMO3 or splMO4 at 4 ng and 6 ng did not produce detectable phenotypic changes (Fig. 3.13B; data not shown), whereas injection at higher dose (8 ng) produced similar but milder phenotype as *crtc1* splice morphants. The milder defect in *crtc1b* splice morphants as compared to *crtc1* morphants suggested that knocking down *crtc1* is more detrimental as compared to *crtc1b*. Preliminary double injection of splMO1+splMO2 (3ng each, total 6ng) or splMO3+splMO4 (3ng each, total 6ng) suggested that they produce similar phenotype as single morphants (data not shown).
Figure 3.12 Phenotype changes in *crtc1* splice morphants at different doses of MO

(A) The wild-type phenotype of uninjected embryos at 48 hpf was compared to morphants injected *crtc1* splice MO 2 (splMO2) at 4 ng (B) and 6 ng (C). The morphants shown here were selected based on observable phenotypic changes (as a subset) from a population of injected embryos, which showed a variable penetrance to the above abnormal phenotype. Red scale bar = 200 µm.

Figure 3.13 Phenotype changes in *crtc1b* splice morphants at different doses of MO

(A) The wild-type phenotype of uninjected embryos at 48 hpf was compared to morphants injected with *crtc1b* splMO3 at 4 ng (B) and 8 ng (C). The morphants shown here represent a population of injected embryos with variable penetrance of the abnormal phenotype. Red scale bar = 200 µm.
3.4.2. Translation blocker

Preliminary assessment of the effect of either \textit{crtc1} or \textit{crtc1b} translation blocker on zebrafish was carried out. Injection of 6 ng anti-\textit{crtc1} tMO caused a phenotypic change in the \textit{crtc1} morphants. The morphants showed an abnormal body curvature bent ventrally. By contrast, injection of anti-\textit{crtc1b} tMO at 6 ng did not cause similar abnormal body curvature; nor did it provoke other observable phenotypic changes. Subsequent dose-response studies showed similar results (Appendix 3). The relatively subtle phenotypic changes in \textit{crtc1b} morphants could be due to compensation (or “rescue”) by \textit{crtc1} given that \textit{crtc1} and \textit{crtc1b} are closely related and share similar spatiotemporal expression patterns. If so, \textit{crtc1b} is clearly less effective in compensating for the loss of \textit{crtc1} in \textit{crtc1} morphants. This distinction could be partly explained if we consider differences in exon usage during early development (see section 3.1). Crtc1b lacks amino acids encoded by exon-11 which is selectively spliced out during maternal transcription, whereas Crtc1 retained the homologous amino acid sequence throughout embryonic development. The above remains speculative and would need further experiments for clarification. In light of the lack of significant observable effects in \textit{crtc1b} morphants, subsequent experiments were carried out using the \textit{crtc1} translation blocker.

3.4.2.1. \textit{crtc1} tMO1 reduced Crtc1-flag expression in electroporated HEK293T cells

To test the effect of translation blocker on the translation of \textit{crtc1} transcript, \textit{crtc1} translation blocker 1 (or tMO1) was injected into 1-cell stage embryo and Western analysis was done on the 24 hpf embryo lysate prepared according to Waskiewicz et al. (2001). \textit{crtc1} tMO1 was designed to hybridize to transcript region spanning -4 (relative to ATG) to +21 (see Fig. 3.19E). Immunodetection with goat polyclonal antibody against C-terminus of human CRTC1 failed to detect the endogenous proteins corresponding to either Crtc1 or Crtc1b. In comparison, immunodetection with antibody against the first 42 amino acids at the N-terminus of human CRTC1 detected multiple bands on Western blot. The latter antibody was expected to detect all the CRTC family members (e.g. Crtc1, Crtc2 and Crtc3) which share very high sequence conservation at the N-terminus. However, there was no observable
difference in the intensity of a band corresponding to the predicted size of endogenous Crtc1 (or Crtc1b) in morphant and control embryos (data not shown; reported in Fang, 2012). The failure to discern the translation blocking effect of the MO was mainly due to a lack of antibody specific to Crtc1 only. In order to circumvent this limitation, I used a plasmid expressing Crtc1 fused to a FLAG epitope to test the tMO1 knockdown effect in both HEK293T cells and zebrafish embryos. The crtc1 cDNA retained the endogenous 5’ untranslated region (5’ UTR) consisting of the tMO1 binding site.

In HEK293T cells electroporated with both crtc1 tMO1 and plasmid expressing Crtc1-flag, there was a reduction in the translated protein as detected by Western analysis using antibody specific to the FLAG epitope. This was in comparison to the control HEK293T cells electroporated with both a standard control MO and the above plasmid (Fig. 3.14A-C). The standard control MO only targeted human beta-globulin intron mutation and served as a negative control in subsequent injections. Increasing the amount of tMO1 used in electroporation further reduced the translated product of the Crtc1-flag protein >8 fold (Fig. 3.14B). This suggested that tMO1 was able to block the translation of Crtc1-flag in cultured cells. To test if tMO1 was able to give a similar effect in zebrafish embryo, I co-injected tMO1 and in vitro synthesized capped and polyadenylated RNA encoding Crtc1-flag into 1-cell stage embryos. At 24 hpf, the embryos were lysed and analysed by Western analysis. Co-injecting tMO1 with the RNA reduced the translated product to half as compared to control MO injected embryos (Fig 3.15). The above results suggested that tMO1 was able to disrupt the translation of crtc1-flag mRNA in both cultured cells and zebrafish embryos.
Figure 3.14  Translation blocker (tMO1) reduced expression of Crtc1-flag in HEK293T cells

(A) HEK293T cells were co-electroporated with plasmid expressing Crtc1-flag along with either control MO or crtc1 tMO1. The amount of each component is shown. The plasmid expressing Crtc1-flag retained the tMO1 binding site upstream of the start codon. (B) Western blot analysis indicated a reduced expression of Crtc1-flag in the presence of tMO1 as compared to control MO and this was further reduced with increasing amount of tMO1 added. (C) The relative abundance of Crtc1-flag was normalized to Actb expression using ImageJ software.

Figure 3.15  Translation blocker (tMO1) reduced the expression of Crtc1-flag in 24 hpf zebrafish embryo

Zebrafish embryos were co-injected with 300ng of capped-and-polyadenylated RNA expressing Crtc1-flag alongside 6ng of either control MO or tMO1. Western analysis indicated a reduction of Crtc1-flag expression when embryos were co-injected with tMO1 as compared to control. The abundance of Crtc1-flag was normalized to Actb expression using ImageJ software and expressed as relative units. The result is representative of two independent experiments.
3.4.2.2. **crtc1 tMO1 morphant phenotype**

In a preliminary experiment, 6 ng of tMO1 was used to analyse the effect of **crtc1** knockdown in zebrafish embryo (see section 3.4.2). This provided a glimpse of the resultant mutant phenotype, noticeably a consistent ventrally directed body curvature (also known as “curly-tail-down”). The degree of the curve increased proportionately with degree of severity and these morphants were subsequently grouped with respect to the severity of the body curvature as either “mild” or “severe” (Fig. 3.17B and C).

In order to determine the MO dosage that was sufficient to cause a mutant phenotype without adverse non-specific effect, a finer dose-response experiment was performed. Embryos were injected with 2, 4, 6 or 8 ng of tMO1 per embryo. For comparison, control embryos were injected with 6 ng of control MO. By relying on gross observation, morphants injected with 6 ng of MO displayed a reasonably high penetrance for the curly-tail-down morphology (~ 50% morphants) with minimal lethality, while injection at 4 ng or less was insufficient. Higher dosage at 8 ng of tMO1 caused some lethality at 48 hpf. Meanwhile, control embryos injected with 6 ng showed a predominantly wild-type phenotype (Fig. 3.16A-E). For subsequent injections, 6 ng of tMO1 was used. This was based on the high penetrance of the curly-tail phenotype afforded by this dosage as well as the low lethality of the morphants.

Further analysis on the **crtc1** morphants using a 6 ng injection of tMO1 showed that there are several distinguishing features. Apart from the curly-tail-down phenotype, **crtc1** morphants displayed a shorter body length than control (Fig. 3.17A-C, E). Furthermore, the eye size in morphants was reduced as compared to control (Fig. 3.17A-C, F) and similarly, the brain appeared smaller in morphants than in controls. Surprisingly, all injected embryos showed a curly-tail-down phenotype with different degrees of severity (Fig. 3.17D). This was different in earlier injections wherein >50% injected embryos showed curly-tail-down while there were some morphants with wild-type phenotype. The difference could be due to variable susceptibility of embryos from different mating to the MO (e.g. “batch effect”). Another explanation could be technical due to an improvement in my injection skills over the course of my work.
In a dose-response experiment to determine sufficient dosage to elicit a phenotypic change while minimizing non-specific side-effects, embryos were injected with (B) 2 ng, (C) 4 ng, (D) 6 ng, and (E) 8 ng of crtc1 tMO1. These were compared to embryos injected with 6 ng of control MO. Injection at 6 ng of tMO1 gave the highest penetrance of the curly-tail-down phenotype with acceptable levels of side-effects (lethality). Embryos injected with control MO exhibited a wild-type phenotype. Red scale bar = 500 µm.
Figure 3.17  Phenotype of crtc1 tMO1 morphants

Zebrafish embryos injected with 6 ng of crtc1 tMO1 displayed abnormal body curvature pointing ventrally (or curly-tail-down). The curly-tail-down phenotype could be classed into (B) mild and (C) severe based on the degree of curvature in comparison to (A) wild-type phenotype. (D) In three independent experiments, injection of 6 ng of tMO1 produced all morphants with curly-tail-down phenotype with variable severity. “Others” (represented as purple bar) denotes non-specific phenotype changes in control embryos (either uninjected or control MO injected embryos). (E) Body length (green dotted line) of morphants was significantly reduced as compared to embryos injected with 6 ng of control MO. (F) Eye size (area demarcated by dotted green line) in morphants was significantly reduced in morphants in comparison to embryos injected with control MO. **** denotes p < 0.0001 and error bar represents standard deviation of number of embryos measured (n) from two independent experiments. Scale bar = 200 µm.
3.4.2.3. Apoptosis in crtc1 morphants

Similar to crtc1 splice morphants at 24 hpf, there was an indication of apoptosis in the brain region as assessed by the presence of an opaque region assumed to be apoptotic tissue in 24 hpf of tMO1 morphants (compare Fig. 3.18C to A and B, upper panels). In order to test this further, Acridine Orange (AO) staining of live embryos showed that AO stained cells were normally present in both uninjected and control injected embryos. These cells were highly labelled in the nervous system, e.g. brain and the neural tube (Fig. 3.18A and B, bottom panels). When compared to crtc1 morphants, it was difficult to determine confidently if there was any difference in morphants and the controls (comprising uninjected and control MO embryos). This was due to the usage of fluorescein tagged MO resulting in the masking of the AO signal (Ex/Em = 503/530 nm) by the fluorescein signal (Ex/Em = 494/521 nm). In the neural tube where fluorescein label was less obscuring, a comparison of AO signal in the morphants did not show an elevated AO signal (assessed by punctate green signals) when compared to the controls (Fig. 3.18A-C, bottom panels). Apoptosis level was also determined by qRT-PCR. qRT-PCR detection of p53 downstream target such as cyclin-dependent kinase inhibitor 1A (cdkn1a or p21) gene (Vousden and Lu, 2002) was performed. There was an approximately 4 fold upregulation of cdkn1a in the morphants as compared to control embryos (Fig. 3.18D).

3.4.2.4. The crtc1 morphant phenotype is independent of p53 function

In order to exclude the possibility that non-specific cell death caused by tMO1 injection was responsible for the observed phenotype, I co-injected a translation-blocking MO against p53. The rationale is based on Robu et al. (2007) who injected the p53 tMO alongside an experimental MO to distinguish “non-specific” apoptosis from a true developmental defect caused by bona fide MO mediated gene knockdown.
Apoptosis induced by MO in *crtc1* morphant

(A) and (B) upper panels represent bright-field images of uninjected and control MO injected embryos respectively at 24 hpf. Both uninjected and control MO injected embryos served as control. By contrast, there were several opaque tissue in the brain region of morphant (C, upper panel) whereas, they were absent in the control embryos. (A-C, lower panels) Acridine orange (AO) staining suggested that apoptotic cells were present in the controls but it did not confidently suggest any difference in morphant and controls. (D) Quantitative RT-PCR result indicates 4 fold upregulation of *cyclin-dependent kinase inhibitor 1A* (*cdkn1a* or *p21*) in 24 hpf control (injected with con MO) and morphant embryos. *cdkn1a* is a pro-apoptotic gene. Error bar represents four technical replicates (n=4) from two independent experiments; *** denotes p < 0.001. Red scale bar = 100 µm.
Morphants co-injected with 6 ng of crtc1 tMO1 and 2 ng of p53 tMO displayed the curly-tail-down phenotype comparable to morphants without p53 tMO (data not shown), whereas morphants co-injected with 6 ng of control MO and 2 ng of p53 tMO presented a wild-type phenotype. This suggested that the curly-tail-down phenotype was not mediated by non-specific apoptotic events. Moreover, the body length, brain and eye size were similarly affected in morphants with (or without) p53 tMO (data not shown). The use of 2 ng of p53 tMO was based on a dose-response of this MO alongside 6 ng of crtc1 tMO1 which elicited less toxicity to the embryo (data not shown). More than 2 ng of p53 tMO caused some toxicity in 6 ng crtc1 tMO1 background.

3.4.2.5. An independent and non-overlapping morpholino, crtc1 tMO2, elicited a similar phenotype

In order to determine the specificity of the crtc1 knockdown phenotype, another non-overlapping translation blocker, tMO2 (spanning -30 to -6, relative to ATG of crtc1 transcript), was used for the injection (Fig. 3.19E). Following co-injection with 6ng of crtc1 tMO2 and 2ng of p53 tMO, morphants at 48 hpf exhibited a curly-tail-down phenotype comparable to tMO1 morphants (Fig. 3.19B-D). In comparison, embryos co-injected with 6 ng of control MO and 2 ng of p53 tMO showed a wild-type phenotype (Fig. 3.197A, D). This suggested that the curly-tail-down phenotype was specific to crtc1 knock down, and that tMO1 or tMO2 was specific in mediating this gene knockdown. Considering that crtc1 tMO1 with or without p53 tMO produced comparable phenotypes, I concluded that the phenotypic consequences of crtc1 knockdown are specific and due to loss of crtc1 function. Subsequent analyses focused on crtc1 tMO1 in either the presence or absence of p53 tMO.

3.4.2.6. Curly-tail-down phenotype was partially rescued with RNA expressing Crtc1-flag

If knocking down crtc1 function in zebrafish embryos produced a specific morphant phenotype, then restoring crtc1 activity by injecting mRNA encoding Crtc1 should
reverse (or ameliorate) the mutant phenotype. To test this, a “rescue” experiment was performed, in which tMO1-resistant, capped, and polyadenylated RNA encoding Crtc1-flag was co-injected with tMO1.

Figure 3.19 Comparable curly-tail-down phenotype in non-overlapping tMO2 injected morphants

Embryos injected with (B) tMO1 and non-overlapping (C) tMO2 against crtc1 produced a similar curly-tail-down phenotype. Meanwhile, embryos injected with (A) control MO showed predominantly wild-type phenotype. (D) Both tMO1 and tMO2 produced high penetrance of the curly-tail-down phenotype (n > 50 embryos per condition, and observed in two independent experiments). (E) cDNA sequence of crtc1 with the first base of start codon (ATG, in capital letters) denoted as +1 and the relative position of tMO1 and tMO2 binding sites indicated. Red scale bar = 500 µm.
Both “tMO1-susceptible” and “tMO-resistant RNA” were transfected in HEK293T and the corresponding Crtc1-flag was detected by using antibody against FLAG in Western analysis (Fig. 3.20A and B). The tMO1-susceptible RNA possessed the tMO1 binding site whereas tMO1-resistant RNA lacked this binding site due to mutation of several bases (Fig. 3.20C). The dosage of the mRNA used was derived from a dose-response experiment and 200 pg was found to minimize side-effects (cyclopia; data not shown).

Morphants injected with the tMO1-resistant mRNA encoding Crtc1-flag displayed a partial rescue of the curly-tail-down phenotype (Fig. 3.21). The frequency of the severe form of the phenotype dropped significantly when morphants were injected with crtc1 mRNA. Similarly, the total number of embryos with curly-tail-down showed significant reduction.

![Figure 3.20](image)

Figure 3.20 Capped and polyadenylated RNA expressing Crtc1-flag and “MO-resistant” Crtc1-flag in HEK293T cells

(A) HEK293T cells were transfected with either RNA expressing Crtc1-flag or “MO-resistant” RNA expressing Crtc1-flag by using Lipofectamine 2000 transfection kit. (B) Western analysis detected both the Crtc1-flag proteins derived from “tMO1 susceptible” and “tMO1 resistant” RNA (red arrowhead). Untransfected cells did not show the corresponding protein band. The blot was re-probed for ActB acting as a loading control. (C) “tMO1 resistant” RNA is devoid of tMO1 binding site due to several bases mutation.
Figure 3.21  RNA expressing tMO1-resistant Crtc1-flag partially rescued the mutant phenotype of tMO1 morphant

Co-injection of capped and polyadenylated RNA expressing Crtc1-flag along with tMO1 partially rescued the curly-tail-down phenotype in morphant. The RNA was designed with mutated tMO1 binding site and therefore expected to prevent tMO1 binding. ** denotes p < 0.01 and n.s. = not significant. Error bar = standard deviation of three independent experiments.

3.4.3. Focus on translation morphants

In the previous section, I have shown that knocking down Crtc1 function by translation blocker produced a consistent and penetrant phenotype with identifiable curly-tail-down morphology. In comparison, *crtc1* splMO morphants displayed a rather pleiotropic phenotype with overlapping defects. Such difference could be caused by the different mode of action in these MOs. For subsequent analyses, I have decided to use translation blockers for reasons to be discussed in section 4.3.
3.5. In situ hybridization analysis

3.5.1. Eye development is affected in crtc1 morphants

Gross observation of crtc1 morphants indicated that they have smaller eyes than controls (Fig. 3.17). The smaller eye phenotype could be indicative of a defect in the lens, retina or both lens and retina development. To test this, in situ hybridization was used to determine the expression of several genes important for lens and eye development, namely islet1, neurod4, olig2, meis1 and meis2b (Elshatory et al., 2007b; Hafler et al., 2012; Inoue et al., 2002; Zhang et al., 2002).

The LIM/homeobox family member islet 1 (isl1) encodes a transcription factor important for specification of primary neurons (Korzh et al., 1993; Pfaff et al., 1996; Tanaka et al., 2011), and in retinogenesis (Elshatory et al., 2007a; Pan et al., 2008) among other roles. In 24 hpf embryos, isl1 expression in crtc1 morphants was comparable to control embryos. isl1 RNA was readily detected in the primary neurons (e.g. Rohon-Beard sensory neuron and motor neurons) of the embryonic spinal cord, in the telencephalon, diencephalon and epiphysis of the forebrain region, cranial nerves of the hindbrain, and pancreas, but not yet in the eye and faintly in the hatching gland (Fig. 3.22A and B). There was a slight increase of isl1 expression in the midbrain region of some morphants, but this expression profile was detected in some control embryos as well.

In control embryos at 48 hpf, isl1 was detected in the forebrain, midbrain, and hindbrain region and its expression was also maintained in the spinal cord and pancreas in control embryos (Fig. 3.23A upper and lower panels). crtc1 morphants expressed isl1 in the above structures as well, but at a distinctly lower levels (Fig. 3.23B in upper and lower panels). Furthermore, isl1 expression in the laminated retina was significantly reduced in morphants as compared to control embryo. This was assessed by measuring the size of the isl1 expression domain in the retina (compare Fig. 3.23A and 3.23B; tabulated in 3.23D). Reduced retinal expression of isl1 in the morphant was rescued when crtc1 tMO1 was co-injected with 200 pg of mRNA encoding Crtc1-flag (compare Fig. 3.23B and 3.23C). The isl1 expression domain increased significantly in rescued embryos as compared to morphants, although the
expression level was still below that of control embryos (tabulated in Fig.3.23D). Perturbation of *isl1* in *crtc1* morphants could explain the small-eye phenotype. This is considering that Isl1 is important for proper establishment of the ganglionic cell layer (GLC) and the inner nuclear layer (INL). It is also important for proper development of the optic nerve and retinal axon pathfinding. Inactivation of Isl1 has been shown to cause apoptosis in GCL, probably due to a retinotectal development defect (Pan et al., 2008).

![Figure 3.22](image.png)

**Figure 3.22** *isl1* expression in 24 hpf morphant and control embryo in wholemount and flatmount *in situ* hybridization (ISH) stain

(A) *isl1* expression in 24 hpf control MO injected embryo in wholemount (upper panel, lateral view) and flatmount (lower panel, dorsal view) is detected in telencephalon (T), diencephalon (D), epiphysis (E), trigeminal nerve (tri), other cranial nerves of the hindbrain (HB), primary neurons of the spinal cord (SC), pharyngeal arches (pha) and in the pancreas (Pan). A comparable expression profile was observed in (B) *crtc1* morphants. Red arrowhead demarcates the midbrain-hindbrain border. Red scale bar = 100 μm.
Figure 3.23  *isl1* expression in 48 hpf morphant and control embryo in wholemount ISH stain

(A-C) 48 hpf embryos shown in dorsal (upper panels) and lateral (lower panels) views. (A) *isl1* expression in an uninjected embryo as control. *isl1* expression in uninjected and control MO-injected embryos is comparable (data not shown). (B) *isl1* expression in *crtc1* morphant and (C) morphant rescued with 200 pg of RNA encoding Crtc1-flag. The expression domain of *isl1* in the eye field of the embryos was measured from the dorsal view within the area demarcated by the green dotted line (upper panels). (D) The size of *isl1* expression domain in the eye field (of both left and right eyes) was measured in square micrometre in control, morphant and rescued embryos. **** denotes p < 0.0001, ** denotes p = 0.0048, and error bar represents standard deviation from number of embryos analysed (n). Red scale bar = 100 µm.
NeuroD4 is a bHLH transcription factor essential for the development of several retinal cell types. Similar to other bHLH transcription factors, NeuroD4 cooperates with other transcription factors (especially homeodomain proteins) for specification of specific retinal cell types (Inoue et al., 2002). In 24 hpf crtc1 morphants, 5/7 embryos showed reduced or absent neurod4 expression in the retina as compared to control embryos (compare Fig. 3.24A to 3.24B). Such loss was not completely recovered in 48 hpf morphants which displayed a smaller neurod4 expression domain (5/7 of morphants; compare Fig. 3.25A to 3.25B). MO-induced apoptosis mediated by upregulation of p53 has been suggested to cause a non-specific brain phenotype (Robu et al., 2007). To test whether p53 plays a role in the observed reduction in neurod4 expression, crtc1 tMO1 was co-injected with p53 tMO. p53 tMO co-injection ameliorated neurod4 expression of 24 hpf morphants as shown by a recovery of neurod4 expression in the retina (compare Fig. 3.26A and 3.26B). Although, p53 tMO co-injection rescued 24 hpf morphant, the MO was unable to rescue the defect seen at later stages (compare Fig. 3.26C and 3.26D). Thus, 48 hpf morphants co-injected with p53 tMO still showed a reduced neurod4 expression domain as compared to control embryos (6/10 of morphants, Fig. 3.26D). This suggested that the reduced neurod4 in the brain and eye region is specific to crtc1 knockdown and not due to non-specific apoptosis.

In this experiment, I have used uninjected embryos as controls to similar extent as control MO-injected and control MO co-injected with p53 tMO. They have been shown to produce consistently similar in situ hybridization results with other RNA probes used (data not shown). Furthermore, quantitative RT-PCR demonstrated that the diagnostic marker del113 p53 which is indicative of unspecific apoptosis produced by MO (Robu et al., 2007) was expressed at low level in the three groups (Appendix 7).
Figure 3.24  *neurod4* expression in 24 hpf morphants and control embryos in wholemount ISH stain

(A) *neurod4* expression in control MO injected embryo visualized laterally (left panel) and dorsally (right panel). This is compared to (B) *crtc1* morphant. In morphants, 7/8 embryos exhibited reduced *neurod4* expression in the retina (asterisk) as compared to control. OB = olfactory bulb, R = retina. Red scale bar = 100 µm.

Figure 3.25  *neurod4* expression in 48 hpf morphant and control embryos in wholemount ISH stain

(A) *neurod4* expression in 48 hpf control MO-injected embryo visualized laterally (left panel) and dorsally (right panel). This is compared to (B) *crtc1* morphant. In 5/7 of morphants, *neurod4* was reduced in the brain region and the eye field. Red scale bar = 100 µm.
Figure 3.26 *neurod4* expression in 24 and 48 hpf morphants co-injected with *p53* tMO and uninjected control embryos in wholemount ISH

Embryos at 24 hpf (A-B) and 48 hpf (C-D) shown in lateral view (upper panels) and dorsal view (lower panels). (A) *neurod4* expression in 24 hpf uninjected control embryo is compared with (B) *crtc1* morphant co-injected with *p53* tMO. (C) *neurod4* expression in 48 hpf uninjected control embryo is compared with (D) morphant co-injected with *p53* tMO. *p53* co-injection in 24 hpf morphant rescued the reduced *neurod4* expression phenotype in morphant; however, at later stage of 48 hpf, 6/10 of embryos still exhibited reduced *neurod4* expression in the brain and eye region as compared to control embryos. Scale bar = 100 µm.
Olig2 is a basic helix-loop-helix (bHLH) transcription factor expressed in retinal progenitor cells (RPCs) at the outer neuroblastic layer (ONBL) but not detected in post-mitotic cells of the eye such as retinal ganglion cells (RGCs), amacrine cells (ACs), bipolar cells (BPs), Muller glia (MG) and photoreceptors (PRs). Olig2 is believed to be important for maintenance of stemness in retinal progenitor cells, which is equivalent to its role in motor neuron progenitors (pMN) in the embryonic spinal cord (Hafler et al., 2012; Shibasaki et al., 2007) and subsequent specification of cell types at later stage could be caused by combinatorial interaction with other transcription factors. The expression of olig2 was detected in the forebrain, the midbrain, the hindbrain, and the neural tube region (likely to be in pMN domain) and faintly in the eye. In 24 hpf morphant, although there was a reduced expression of olig2 in the brain region of some morphants, olig2 expression in most morphants were comparable to control embryos (Fig. 3.27A and 3.27B). At 48 hpf, morphants displayed a distinctive reduction of olig2 expression in the brain and eye region when compared with controls (6/10 of morphants; compare Fig. 3.28A and 3.28B). Reduced expression of olig2 in the eye field is consistent with reduced expression of isl1 and neurod4 as all three genes have been implicated in retinogenesis (Elshatory et al., 2007b; Hafler et al., 2012; Inoue et al., 2002).

Figure 3.27 olig2 expression in 24 hpf morphant and control in wholemount and flatmount ISH

(A) olig2 expression in 24 hpf control MO injected embryos viewed laterally (upper panel) and in flatmount (lower panel). This was compared to (B) crtc1 morphant. Red scale bar = 100 µm.
Figure 3.28  \textit{olig2} expression in 48 hpf morphant and control in wholemount ISH

(A) \textit{olig2} expression in 48 hpf control MO injected embryo viewed laterally (upper panel) and dorsally (lower panel). This was compared to (B) \textit{crtc1} morphant. 6/10 of morphants displayed reduced \textit{olig2} expression in the brain and eye region as compared to control. Red scale bar = 100 µm.

\textit{Meis} gene family is important for eye development, especially in the maintenance of retinal progenitor cells. The downregulation of \textit{Meis1} and \textit{Meis2} are required for retinal cell differentiation. (Bessa et al., 2008; Conte et al., 2010; Erickson et al., 2010; Heine et al., 2009). Perturbation of Meis expression would then be expected to affect eye development. In \textit{crtc1} morphants, the pattern of \textit{meis2b} expression was unperturbed as judged by \textit{meis2b} RNA expression in 24 hpf morphants as compared to control (compare Fig. 3.29A and 3.29B). Its expression was detectable in the forebrain, midbrain, hindbrain and the spinal cord as well as in non-neuroepithelial tissue such as the somites/myotomes. \textit{meis2b} expression in the eye field at this stage was faintly detectable in both controls and morphants.

At 48 hpf, \textit{meis2b} expression domain in the eye was significantly reduced in morphants versus controls (Fig. 3.30A-C). By contrast, \textit{meis2b} expression in the brain region and liver was unaffected (Fig. 3.30A and 3.30B). In addition, there was reduced expression in the anterior pharyngeal arch region.
Figure 3.29  *meis2b* expression in 24 hpf control and morphant (co-injected with *p53* tMO) in wholemount and flatmount ISH

(A) *meis2b* expression in 24 hpf control MO and *p53* tMO co-injected embryo viewed laterally (left panel) and flatmount (right panel). This is compared to (B) *crtc1* morphant co-injected with *p53* tMO. Scale bar = 100 µm.

Figure 3.30  *meis2b* expression in 48 hpf control and morphant (co-injected with *p53* tMO) in wholemount ISH

(A-B) *meis2b* expression in 48 hpf control MO and *p53* tMO co-injected embryo viewed laterally (A) and dorsally (B). This is compared to (C-D) *crtc1* morphant co-injected with *p53* tMO. (E) Combined area of *meis2b*-expressing cells in the eye (demarcated by green dotted line) was measured in control and morphant. **** denotes p < 0.0001, and error bar represents standard deviation from number of embryos analysed (n). Scale bar = 100 µm.
Meis1 has been shown to be involved in the patterning, as well as maintenance of progenitor cells in the eye (Bessa et al., 2008; Erickson et al., 2010). Similar to meis2b, meis1 expression pattern is not perturbed in crtc1 morphants at 24 hpf. meis1 expression in the olfactory bulb, retina, midbrain, hindbrain and spinal cord was detected in both morphant and control (Fig. 3.31). At 48 hpf, crtc1 morphants showed perturbed meis1 expression relative to controls. meis1 expression in both the olfactory bulb and the retina was reduced, whereas the lens, ventral structures (e.g. pronephric duct or branchial arches), hindbrain and spinal cord were unaffected (Fig. 3.32A and 3.32B). Together, these results implicate multiple Meis family members in crtc1-mediated control of retinogenesis.

**Figure 3.31**  *meis1* expression in 24 hpf control and morphant in wholemount and flatmount ISH

(A) meis1 expression in 24 hpf control MO injected embryo viewed laterally (left panel) and flatmount (right panel). meis1 expression is detected in olfactory bulb, retina, midbrain, hindbrain and the spinal cord. This is compared to (B) crtc1 morphant. Red scale bar = 100 µm.
3.5.2. Brain growth/development affected in *crtc1* morphants

Based on the gross morphological assessment of *crtc1* morphants, most displayed a smaller brain and eyes as compared to controls and this phenotype positively correlates with the severity of the curly-tail-down phenotype. This could be due to the effect of overall stunted growth of the embryo, considering that morphants are shorter, and smaller than control embryos (section 3.4.2.2). In order to measure the extent of reduction in the brain size, *in situ* hybridization staining of several proneural genes was performed.

In 24 hpf morphants, the patterning of proneural markers such as *islet1*, *neurod4* and *olig2* was comparable to control embryos, suggesting that their expression in the morphant brain was not obviously affected by *crtc1* knockdown (see section 3.5.1). In addition, the patterning of other proneural markers such as *ascl1b* and *nkx2.2a* was likewise unaffected (i.e. no obvious patterning defect), although the expression level of *ascl1b* (but not *nkx2.2a* in Fig. 3.37) in the forebrain, midbrain and hindbrain was reduced in morphants as compared to control embryos (compare Fig.
The reduced ascl1b expression could be rescued by either co-injection of mRNA encoding Crtc1-flag (4/6 rescued morphants; compare Fig. 3.33B and 3.33C) or co-injection of p53 tMO (compare Fig. 3.34A and 3.34B). Co-injection of p53 tMO showed better recovery of ascl1b expression in the brain region of morphants than the rescue with mRNA co-injection (compare Fig. 3.33C and 3.34B).

Figure 3.33 ascl1b expression in 24 hpf control, morphant, and rescued embryos in wholemount ISH

(A) ascl1b expression in 24 hpf control MO injected embryos viewed laterally. This is compared to (B) crtc1 morphant and (C) rescued morphants. 7/12 morphants have reduced ascl1b expression compared to control; and co-injection of RNA encoding Crtc1-flag recovered ascl1b expression in 4/6 of rescued morphants. Teg = anterior tegmentum, HB = hindbrain, SC = spinal cord, and red arrowhead demarcates midbrain-hindbrain border. Red scale bar = 100 µm.

Figure 3.34 ascl1b expression in 24 hpf control and morphants (both co-injected with p53 tMO) in wholemount and flatmount ISH

(A) ascl1b expression in 24 hpf control MO and p53 tMO co-injected embryos viewed laterally (upper panel) and in flatmount (lower panel). This was compared with (B) crtc1 morphant co-injected with p53 tMO. Scale bar = 100 µm.
In 48 hpf morphants, there was a reduced expression level of *isl1*, *neurod4* and *olig2* in the head region (Fig. 3.23, 3.25, and 3.28). Co-injection of RNA encoding Crtc1-flag partially rescued *isl1* expression in morphants (compare Fig. 3.23B and 3.23C). In contrast, co-injection of *p53* tMO did not rescue the reduced expression of *neurod4* in morphants (compare 3.25B and 3.26D). This suggested that the reduced gene expression is specific to *crtc1* knockdown.

Similarly, the expression of *ascl1b* and *nkx2.2a* were reduced in 48 hpf morphants as compared to control embryos (compare Fig. 3.35A and 3.35B, and Fig. 3.39A and 3.39B). Moreover, the expression pattern of *ascl1b* in the morphant (from the dorsal view) was altered such that the elaborate *ascl1b* expression in the posterior tectum and the telencephalon seen in control embryos was reduced in morphants (compare Fig. 3.35A and 3.35B). This was also true for *nkx2.2a* expression in morphants, for example the laterally expanded *ascl1b* expression domain in the telencephalon and midbrain was reduced in size in morphants as compared to controls (Fig. 3.39A and 3.39B). The reduced size of proneural expression domain in the brain seemed to correlate with the reduced head size of morphants, and could provide a means to measure head size. By measuring the width of the *ascl1b* expression domain in the posterior tectum or the width of the *nkx2.2a* expression domain near the diencephalon (see diagram for explanation in Fig. 3.41), a significant reduction in size of *ascl1b* and *nkx2.2a* expression domain could be documented in morphants (Fig. 3.35D, 3.36C and 3.39D). Co-injecting mRNA encoding Crtc1-flag partially, but significantly, restored the size of *ascl1b* and *nkx2.2a* expression domains in morphants as compared to controls (Fig. 3.31D and 3.35D). Co-injecting *p53* tMO ameliorated the phenotype, but there was still significant reduction in the size of *ascl1b* and *nkx2.2a* expression domains in morphants (Fig. 3.36A-C and Fig. 3.40A-C).
Figure 3.35  *ascl1b* expression in 48 hpf control, morphant, rescued embryos in wholemount ISH

(A) *ascl1b* expression in 48 hpf uninjected control embryos viewed laterally (upper panel) and dorsally (lower panel). This was compared to (B) *crtc1* morphant and (C) rescued morphant. (D) The width of *ascl1b* expression domain in the posterior tectum was measured in controls, morphants and rescued embryos. **** denotes p < 0.0001 and error bar represents standard deviation of number of embryos analysed (n). CeP = cerebellar plate, MO = medulla oblongata, OB = olfactory bulb, Pa = pallium, pte = posterior tectum, Teg = tegmentum, red arrowhead demarcates midbrain-hindbrain boundary, and green spotted line represents width of *ascl1b* measured. Red scale bar = 100 µm.
Figure 3.36  *ascl1b* expression in 48 hpf control and morphant (both co-injected with *p53* tMO) in wholemount ISH

(A) *ascl1b* expression in control MO and *p53* tMO co-injected embryos viewed dorsally. This is compared to (B) *crtc1* morphant co-injected with *p53* tMO. (C) The width of *ascl1b* expression in the posterior tectum was measured in controls and morphants. **** denotes p < 0.0001 and error bar represents standard deviation of number of embryos analysed (n). Scale bar = 100 µm.
Figure 3.37  \textit{nkx2.2a} expression in 24 hpf control, morphant and rescued embryos in wholemount ISH

(A) \textit{nkx2.2a} expression in 24 hpf uninjected control embryos viewed laterally. This was compared to (B) \textit{crtc1} morphant and (C) rescued embryo. Red scale bar = 100 µm.

Figure 3.38  \textit{nkx2.2a} expression in 24 hpf control and morphant (co-injected with \textit{p53} tMO) in wholemount ISH

(A) \textit{nkx2.2a} expression in 24 hpf uninjected control embryos viewed laterally (upper panel) and dorsally (lower panel). This is compared to (B) \textit{crtc1} morphant co-injected with \textit{p53} tMO. Scale bar = 100 µm.
**Figure 3.39 nknx2.2a expression in 48 hpf control, morphant and rescued embryos in wholemount ISH**

(A) *nknx2.2a* expression in 48 hpf uninjected control embryos viewed laterally (left panel) and dorsally (right panel). This is compared to (B) *crtc1* morphant and (C) rescued embryo. (D) The width of *nknx2.2a* expression near the diencephalon (green dotted line) was measured in control, morphant and rescued embryo. **** denotes p < 0.0001 and error bar represents standard deviation of number of embryos analysed (n). D = diencephalon, Hy = hypophysis (pituitary gland), Teg = tegmentum, and green dotted line represent measured width of *nknx2.2a* expression domain. Red scale bar = 100 µm.
Figure 3.40  *nkx2.2a* expression in 48 hpf control and morphant (co-injected with *p53* tMO) in wholemount ISH

(A) *nkx2.2a* expression in 48 hpf uninjected control embryo viewed laterally (left panel) and dorsally (right panel). This is compared to (B) *crtc1* morphant co-injected *p53* tMO. (C) The width of *nkx2.2a* expression domain near the diencephalon (green dotted line) was measured in controls and morphants. **** denotes p < 0.0001 and error bar represents standard deviation of number of embryos analysed (n). Scale bar = 100 µm.
Figure 3.41  Measurement of brain size by correlation to width of *ascl1b* and *nkh2.2a* expression domains

The width of *nkh2.2a* expression near the diencephalon and the width of *ascl1b* at posterior tectum were used as landmark for measurement. Red outline corresponds to *nkh2.2a* expression domain, while green outline corresponds to *ascl1b* expression domain. Scale bar = 100 µm.

3.5.3. Other phenotypes

3.5.3.1. Dorsoventral (DV) patterning at gastrula stage affected in *crtcl* morphant

Body proportion is dependent on the patterning at multiple axes. Failure to adhere to these patterning programs can change body shape and structure. For example, DV patterning is tightly and dynamically regulated (Inomata et al., 2013), and defective DV pattern has been shown to cause dorsalized (or ventralized) phenotype in zebrafish due to expansion of dorsal structures at the expense of ventral structures and *vice versa* (Kishimoto et al., 1997). Disturbed DV pattern has been shown to cause defect in the brain (due to smaller neuroectoderm region), notochord and perturbation of left-right (LR) laterality (reviewed in De Robertis and Kuroda, 2004).

In the zebrafish, DV patterning can be assessed by using the ISH method to visualize the *bmp4* expressing domain in the ventral region of shield stage embryos. *bmp4* is expressed at the ventral signalling centre (De Robertis and Kuroda, 2004). Reciprocally, *goosecoid (gsc)* expression provides a marker of the dorsal (organizer) region. Perturbation in DV patterning is expected to affect both *bmp4* and *gsc*
expression domains. In shield stage (6 hpf) crtc1 morphants, the ventral \textit{bmp4} expressing domain was reduced (Fig 3.42D; compare Fig 3.42A and B), while the \textit{gsc} expression domain was expanded (Fig 3.43D; compare Fig 3.43A and B) in relation to controls. However, co-injection of mRNA encoding Crtc1-flag did not rescue this DV patterning defect (Fig 3.42D and 3.43D).

\textbf{Figure 3.42} \textit{bmp4} expression in shield stage (6 hpf) controls, morphants and rescued embryos (co-injected with \textit{p53 tMO}) in wholemount ISH

(A-C) shield stage embryos are shown in animal pole view (upper panels) and lateral view (dorsal to the right; middle panels). Enhanced and pseudocoloured images corresponding to animal pole view are shown at lower panels. (A) \textit{bmp4} expression in ventral region of control MO and \textit{p53 tMO} co-injected embryos compared to (B) \textit{crtc1} morphants and (C) rescued embryos. (D) The angular degree, \(d\), of \textit{bmp4} expression in ventral region was measured in control, morphants and rescued embryos. *** represents \(p < 0.001\), ** represents \(p < 0.01\) and n.s. = not significant. Error bar represents standard deviation of number of embryos analysed (n). Red scale bar = 100 µm.
Figure 3.43  *gsc* expression in shield stage (6 hpf) controls, morphants and rescued embryos (co-injected with *p53* tMO) in wholemount ISH

(A-C) Shield stage embryos are shown in animal pole view (upper panels) and lateral view (dorsal to the right; lower panels). (A) *gsc* expression in dorsal region of control MO and *p53* co-injected embryos compared to (B) *crtc1* morphants and (C) rescued embryos. (D) The angular degree, d, of *gsc* expression in the dorsal region was measured in controls, morphants and rescued embryos. **** denotes p < 0.0001 and *** denotes p = 0.0004. Error bar represents standard deviation of number of embryos analysed (n). Red scale bar = 100 µm.
3.5.3.2. Mild deviation of heart left-right (LR) asymmetry in morphant

Laterality during embryonic development has been attributed to the effect of unilateral fluid propulsion carrying laterality factors (e.g. nodal factors) towards the anterior part of the body (Nonaka et al., 2005; Okabe et al., 2008), but several recent findings suggested that there are more complex schemes at work (reviewed in Vandenberg and Levin, 2013). A useful readout for abnormal specification of LR asymmetry is the assessment of the jogging (or placement) and looping of heart primordia. Heart (or cardiac) jogging is a term used to define the morphogenetic process in which the heart tube/cone is displaced to the left (GO:0003146; http://zfin.org/action/ontology/ontology-search). Heart jogging is dependent on asymmetric Bmp4 or Nodal expression in the heart tube (Chen et al., 1997b; Chin et al., 1997; Lenhart et al., 2013). Normally, heart jogging precedes looping, in which the cardiomyocytes (from the heart tube) are displaced anteriorly and the left. This is then followed by differentiation of these cells and changes in their morphology that drives the heart tube looping to the right. Deviation from these events is considered abnormal. Preferential heart jogging is dependent on differential myocardial cell migration rate to the left (e.g. higher on left than right) and this depends on Nodal and Bmp signals (Lenhart et al., 2013). Zebrafish ENU mutants with abnormal heart left-right asymmetry can be categorized into four phenotypic groups, and one of the groups showed concomitant body shape defects such as curly-tail caused by a probable midline structure defect (Chen et al., 1997b). shha mRNA (which is expressed in the midline) when ectopically injected into chick and zebrafish, respectively, causes randomized heart jogging and looping (Chen et al., 1997b; Levin et al., 1995). However, the exact role of shha or other hedgehog members in asymmetric heart positioning is still unrefined. Retinoic acid signalling is also involved in heart asymmetry (Huang et al., 2011).

In crtc1 morphants, there was a mild deviation from normal heart positioning, in which 11.1% (or 4/36 embryos) showed abnormal heart jogging to the right or centre (as assessed by bmp4 staining; Fig 3.44D; compare Fig 3.44 A, B and C). Furthermore, previous work by Fang (2012) showed that crtc1 morphants exhibited defects in notochord differentiation indicated by persistent Hh signal in the structure as compared to control embryos.
Ciliogenesis is important for determination of LR asymmetry (reviewed in Lessman, 2012). Cilia are also important for Hh signalling (Huang and Schier, 2009). Zebrafish MZovl (jft88 mutation) mutants lacking cilia exhibited LR patterning defects and other signs of ciliopathy, e.g. heart edema and kidney cysts (Huang and Schier, 2009). Moreover, other ciliopathy-related phenotypes include retinal degeneration, cerebral anomalies and otolith defects (Colantonio et al., 2008; Waters and Beales, 2011). In 48 hpf embryos, 46.1 percent of the crtc1 morphants displayed abnormal otolith number (Chi-square p value = 3.5 x 10^{-7}) as compared to 8.1 percent in controls (Fig 3.45D). Embryos normally have two otoliths (Fig 3.45B), whereas embryos with abnormal number of otolith displayed one or three otolith(s) (see inset in Fig 3.45A and B, respectively).

**Figure 3.44**  Heart jogging assessed by bmp4 expression in heart primordium in controls and morphants

Heart jogging/positioning is determined by bmp4 expression in the heart primordium. (A) wild-type heart is positioned to the left while abnormal heart is positioned in the (B) midline and (C) to the right. (D) Percentage of embryos with abnormal heart was tabulated in 24 hpf control MO-injected embryos and crtc1 morphants. Number of embryos assessed (n). Scale bar = 100 µm.
Figure 3.45  Otolith number in controls and morphants

Wild type embryo with two otoliths (B) compared to an embryo with one (A) or three (C) otoliths (see insets). (D) Percentage of embryos with abnormal otolith numbers were tabulated in control and crtc1 morphant populations. **** represents $p = 3.5 \times 10^{-7}$ derived from Chi-square test; $n =$ number of embryos analysed.
3.5.3.3. Convergent movement affected in morphants

Considering that *crtc1* morphants were smaller and shorter in body length than control embryos, it was possible that convergent extension (CE) movements were affected during early development. To test whether convergence movement was affected, embryos at bud stage (10 hpf) were ISH stained for *myod1* expression in lateral mesoderm flanking the midline structure. The rate of convergence was assessed by measuring the width between the left and right *myod1* expression domains in lateral mesoderm (green lines in Fig 3.46A-C, upper panels). As convergence progresses, the distance between the *myod1* expressing domains contracts. I found that the distance between *myod1* expression domains in *crtc1* morphants was greater than controls, suggesting that convergence movements were reduced in the former (compare Fig 6.46A and B upper panels; Fig 3.46D). Convergence movements were restored when morphants were co-injected with mRNA encoding Crtc1-flag (Fig 3.46A-D). *krox20* was co-stained to mark rhombomere 3 (r3) at bud stage (refer to [http://zfin.org/](http://zfin.org/)).

To test whether extension movements were affected, the length of embryo proper from the polster down to the posterior expression domain of *myod1* was measured. At bud stage, the length of the embryo proper in controls, morphants and rescuants was comparable, suggesting that extension movements were unaffected (data not shown). This is surprising considering that morphants showed a convergence defect. A probable explanation for the absence of an extension phenotype could be due to the fact that zebrafish axial mesoderm extension is not solely dependent on convergent movement during gastrulation, but also on additional motive force (reviewed in Rohde and Heisenberg, 2007). Unlike *X. laevis*, zebrafish axial extension persists even though convergence movement is perturbed. The additional motive force appears to be active cell migration of a group of involuting cells towards the animal pole (Glickman et al., 2003; Montero et al., 2005).
Figure 3.46 Convergence movement assessed by width of *myod1*-expressing cells of the lateral mesoderm

(A-C) Bud stage embryos depicted in dorsal view (upper panels) and lateral view (lower panels) in which animal pole is facing up. (A) *myod1* expression in lateral mesoderm and *krox20* expression in r3 of control MO and *p53* tMO co-injected embryos is compared with (B) *crtc1* tMO1 morphant and (C) rescued embryos by co-injection of *crtc1* tMO1 and mRNA encoding MO-resistant Crtc1-flag. (D) The width of *myod1* expression (green line) in left and right lateral mesoderm was measured in control, morphant and rescued embryos. ** denotes p < 0.01, n.s. = not significant, and the error bars represent the standard deviation based on the number of embryos analyzed (n). Scale bar = 100 µm.

3.5.3.4. Neural crest migration and terminal nerve (TN) development is affected in *crtc1* morphants

Trunk neural crest migration can be assessed by following its movement in the dorsal-to-lateral and dorsal-to-ventral paths (Serbedzija et al., 1990). The rate of this migration can then be compared between experimental and control embryos by
staining neural crest specific markers such as *sox10* by *in situ* hybridization. If trunk neural crest migration rate is affected, the *sox10* expression domain that corresponds to the neural crest migratory path in the posterior region will be altered as compared to controls. In addition, other perturbations to neural crest cell development can be assessed by comparing the expression level of *sox10*.

At 24 hpf, the expression level of *sox10* in morphants and control embryos was comparable. The only distinguishing feature between these embryos was that *sox10* expression corresponding to neural crest migration path in the trunk region (highlighted by green line) of morphant showed slight reduction as compared to control embryo (compare Fig 3.47A and B; compare the posterior limit of ventrally migrating neural crest cells shown by arrowheads). The reduced neural crest migration as deduced from the *sox10* expression domain was recapitulated in morphants co-injected with *p53* tMO (compare Fig 3.48A and B). At 48 hpf, *sox10* expression in the trunk and tail region showed an overall reduction (Fig 3.47C-D; Fig 3.49A-B) and this coincided with the completion of crest migration and the transition of multipotent neural crest cells (that expressed *sox10*) to differentiated cells at later developmental stages (Kelsh, 2006). *sox10* expression at the trunk region was indistinguishable in morphant and control embryos at 48 hpf.

However, in the head region of 48 hpf embryos, *sox10* expression at the terminal nerve (TN) in morphants showed reduced expression as compared to control ones (compare Fig 3.47C and D). TN is a cranial nerve and it contains cells that express gonadotropin-releasing hormone (GnRH3) with neuromodulatory role in the forebrain and olfactory region, and hence regulate olfactory related behaviour. These cells are thought to derive from olfactory placodes and cranial neural crest cells (Whitlock et al., 2005). The latter cells express *sox10*. A reduced *sox10* expression in TN would indicate a reduced cranial neural crest cells and hence disruption in TN development. Co-injection of *p53* tMO in morphant embryo could not rescue the reduced *sox10* expression in the terminal nerve (compare Fig 3.49A and B).
Figure 3.47  *sox10*-expression in 24 hpf and 48 hpf control and morphant embryos in wholemount ISH

Embryos at 24 hpf (A-B) and 48 hpf (C-D) are shown in lateral (left panels) and dorsal view (right panels). (A) *sox10* expression in 24 hpf control MO-injected embryos compared to (B) *crtc1* morphant embryos. *sox10* expression in the trunk region is reduced in morphants (green line). (C) *sox10* expression in 48 hpf control embryos compared to (D) morphants. Staining of *sox10* in TN is reduced in morphants. TN = terminal nerve. Red arrowheads demarcate posterior limit of migrating neural crest cells. Red scale bar = 100 µm.
Figure 3.48  *sox10*-expression in 24 hpf controls and morphants (co-injected with p53 tMO) in wholemount ISH

(A-B) 24 hpf embryos viewed laterally (left panels) and dorsally (right panels). (A) *sox10* expression in 24 hpf control MO and p53 MO co-injected embryos compared to (B) *crtc1* tMO1 and p53 MO co-injected morphants. *sox10* expression in the trunk region is reduced in morphants (green line). Red arrowheads demarcate posterior limit of migrating neural crest cells. Scale bar = 100 µm.

Figure 3.49  *sox10*-expression in 48 hpf controls and morphants (co-injected with p53 tMO) in wholemount ISH

(A-B) 48 hpf embryos viewed laterally (left panels) and dorsally (right panels). (A) *sox10* expression in 48 hpf control MO and p53 MO co-injected embryos compared to (B) *crtc1* tMO1 and p53 MO co-injected morphants. The intensity of *sox10* expression in TN is reduced in morphants. Red scale bar = 100µm
3.5.3.5. DV patterning in retina affected in morphant

Cyp26a1 is a metabolic enzyme that converts RA into bio-inactive form and it is involved in RA patterning. It has been suggested that Cyp26a1 acts to adjust proper RA concentration and signalling by acting antagonistically to RA producing retinaldehyde dehydrogenase (Raldh) proteins (Hu et al., 2008). Furthermore, cyp26a1 expression is regulated by RA via the binding of RA receptors to a conserved RA-response element (RARE) in its promoter region (Hu et al., 2008; Loudig et al., 2005; Loudig et al., 2000). In the retina, Cyp26a1 is expressed in a horizontal boundary (or DV boundary) separating the dorsal and the ventral region of retina. The dorsal region expresses Raldh1 (and Raldh2) while the ventral region expresses Raldh3. The DV patterning of the retina is reflected by an RA gradient which is attributed to the differential efficiency of Raldh1 and Raldh3 activity. Ventral Raldh3 is more efficient and hence RA concentration is higher ventrally and this creates a gradient of RA. Although retinal DV patterning might not be dependent on RA signalling (this remains controversial; Molotkov et al., 2006; Sen et al., 2005; Wagner et al., 2000), perturbation of the DV RA pattern could provide clues to a defect in the retinal DV pattern.

In 24 hpf embryos, cyp26a1 expression was detected in the eye, epidermis, pharyngeal arches, spinal cord, trunk and tail region, which was similar in both morphant and control embryos (compare Fig 3.50A and B). At 48 hpf, cyp26a1 expression in the trunk and tail was reduced except at the cloacal region (data not shown). In the head region, cyp26a1 was detected in the five pharyngeal arches, pectoral fin and eye region. cyp26a1 expression in the pharyngeal arches and fin was unaffected in morphant and control embryos (see Fig 3.50C and D). In the eye region, there was an observable difference in cyp26a1 expression. cyp26a1 was expressed near the ciliary marginal zone in control embryos whereas it was more diffuse and present in the retina, optic stalk and periocular region (see Fig 3.50C and D, upper panels). Both control and morphant embryos had been co-injected with p53 tMO. This preliminary result suggested that DV patterning in the eye field might be affected in crtc1 knockdown.
Figure 3.50  *cyp26a1* expression in controls and morphants (co-injected with *p53*) in wholemount ISH

(A-B) 24 hpf embryos viewed laterally, and (Ca and Da) 48 hpf embryos viewed laterally and (Cb and Db) viewed dorsally. (A) *cyp26a1* expression in 24 hpf control MO and *p53* MO co-injected embryos compared to (B) *crtc1* tMO1 and *p53* MO co-injected morphants. (Ca and Cb) *cyp26a1* expression in 48 hpf controls compared to (Da and Db) morphants. The green dotted circle highlights the eye region/field, whereas the green curved line highlight the pectoral fin. Arrowhead marks expression of *cyp26a1* near the ciliary margin, whereas asterisk marks a diffused expression in the eye field. Pha = pharyngeal arches and pec fin = pectoral fin. Red scale bar = 100 µm.
3.5.3.6. Myelination of neurons might be affected in morphants

The reduced brain size of morphants indicated probable perturbation in brain development. There has been a report showing that reduced head size could be attributable to hypomyelination. Igf1 homozygous null mice exhibited reduced brain size and CNS hypomyelination with concomitant reduction of oligodendrocyte number (Beck et al., 1995). Creb protein has been identified as an upstream regulator of Igf1 expression important for proliferation of neural progenitor cells (Choi et al., 2008). Creb might also act downstream of Igf1 via the PKC-PI3K-Akt pathway (Du and Montminy, 1998; Peltier et al., 2007). Oligodendrocytes myelinate neurons in the central nervous system (CNS). This is accomplished in part by expression of *proteolipid protein 1b* (*plp1b*) encoding a transmembrane myelin proteolipid protein. *Plp1b* is important for the myelin sheath formation in both Schwann cells and oligodendrocytes.

In 24 hpf embryos, *plp1b* was detected in the otic vesicle, forebrain, midbrain and hindbrain. The *plp1b* expression level was generally low in morphants and controls and required longer staining time to achieve the higher signal intensity shown here (Fig 3.51A and B). In the ZFIN database, *plp1b* expression is reported as undetectable in 24 hpf embryos (Thisse and Thisse, 2005; http://zfin.org/), whereas *plp:GFP* reporter line showed otherwise (Yoshida and Macklin, 2005). In addition, expression of another myelin sheath protein, *myelin basic protein a* (*mbpa*) gives similar conflicting results (Ke et al., 2008; Thisse and Thisse, 2005). The discrepancy could be due to differences in the RNA probes used for ISH as well as the sensitivity of GFP detection. I found that morphants expressed lower *plp1b* levels in the hindbrain region than control embryos (compare Fig 3.51A and B). At 48 hpf, *plp1b* was detected in the otic vesicle and the anterior cranial nerve region in controls. In morphants, *plp1b* was also detected in otic vesicle but not in the anterior cranial nerve region as compared to controls (compare asterisks in Fig 3.51C and D). Both morphants and controls were co-injected with p53 tMO.
Figure 3.51  *plp1b* expression in controls and morphants in wholemount ISH

(A-B) 24 hpf embryos viewed laterally, and (C-D) 48 hpf embryos viewed laterally (upper panels) and dorsally (lower panels). (A) *plp1b* expression in 24 hpf control MO and *p53* MO co-injected embryos compared to (B) *crtc1* tMO1 and *p53* MO co-injected morphants. (C) *plp1b* expression in 48 hpf controls compared to (D) morphants. HB = hindbrain, OV = otic vesicle, asterisks = anterior cranial nerve region, red arrowheads demarcate midbrain-hindbrain boundary. Red scale bar = 100 µm.
4.1. Spatiotemporal expression of *crtc1* members during embryonic and early larval development

There are two genes belonging to the *crtc1* family, namely *crtc1* and *crtc1b*. *crtc1* was deduced from automated computational analysis whereas *crtc1b* was identified through a cDNA clone (http://www.ncbi.nlm.nih.gov/). The expression profiles of both members were similar, except that *crtc1* was also expressed in the caudal fin fold at 48 hpf, while *crtc1b* was not. Both were ubiquitously expressed in early development but at later stages they were found to be more strongly expressed in the brain region (section 3.1). Their expression profiles were validated by semi-quantitative RT-PCR and interestingly there were alternative splice products exclusively detected for *crtc1b* (section 3.1; Fig 3.3). Maternal expression excluded exon 11 of *crtc1b* whereas zygotic expression included it. These variants were denoted as early and late species. The amino acid sequence derived from exon 11 (simplified as “Ex11_domain”) is highly conserved in other organisms (Appendix 1). Functional characterization of early and late isoforms in a CRE luciferase reporter assay showed that they are comparable in coactivator activity, suggesting that Ex11-domain has minimal effect in this aspect (section 3.2). Previously, CRTCs have been reported to possess splicing activity (Amelio et al., 2009) and considering that Ex11_domain is close to the reported splicing domain, it is possible that it could play a role in RNA splicing (Appendix 1, amino acid sequence highlighted in blue box). Similar to Crtc1b, Crtc1 possessed co-activator activity (section 3.2) suggesting that both CRTC1 members are *bona fide* genes.

*Crtc1* spatiotemporal expression patterns in other vertebrate models are unavailable (http://xenbase.org/; http://geisha.arizona.edu/geisha/; http://www.informatics.jax.org/) and hence no comparative analysis could be made. However, microarray analysis of human and mouse *CRTC1* expression in adult tissues showed similar spatial expression as zebrafish larvae, in which they are ubiquitously expressed but with higher levels in brain (section 3.1; http://biogps.org; Conkright et al., 2003; Wu et al., 2013). In contrast, *CRTC2* and *CRTC3* are expressed more abundantly in other peripheral tissues (Conkright et al., 2003). In invertebrates such as...
D. melanogaster, there is one reported Crtc gene (Gene Accession AAF49313; or FlyBase ID DmeI_CG6064), which is maternally expressed and found ubiquitously in many tissues (http://flybase.org), and have been shown to play a role in long term memory production in the mushroom bodies (homologous to vertebrate hippocampus) (Hirano et al., 2013). In C. elegans, crtc-1 which is a sole CRTC family member is expressed in the neurons of head and tail. Its expression is also found throughout the intestine (Mair et al., 2011).

4.2. Coactivator activity of Crtc1 and Crtc1b

Crct1 produces a higher level of activation at the CRE element that drives firefly luciferase expression than Crtc1b, suggesting that there might be a difference in the coactivator activity between Crtc1 and Crtc1b proteins (section 3.2; compare Fig 3.5B and 3.6B). Alternatively, the distinction could also be due to the difference in Crtc1 and Crtc1b protein expression levels. This is considering that cloned DNA construct encoding Crtc1-flag has no 3’UTR, whereas DNA construct encoding Crtc1b retained 88 nucleotide of 3’UTR. Transcript stability and turnover are governed by the 3’ UTR, and this region is targeted by miRNAs involved in mRNA decay via the activity of RNA-induced silencing complex (RISC; reviewed in Valencia-Sanchez et al., 2006). The targeting of the 3’ UTR of crtc1b mRNA by miRNAs has not been studied thus far, but does not preclude its role in regulating crtc1b mRNA degradation. In addition, the 5’UTR of mRNA has been shown to regulate both transcript stability and translation initiation (Araujo et al., 2012; Elfakess and Dikstein, 2008; Wilusz et al., 2001). A comparison of 5’UTR of crtc1-flag and crtc1b mRNA sequence showed a divergent nucleotide sequence upstream of -14 (relative to AUG), whereas nucleotide sequence at -13 to +5 is conserved in crtc1-flag and crtc1b. How the 5’UTR might influence crtc1-flag and crtc1b protein translation is currently unknown. Preliminary Western analysis to determine the relative expression of zebrafish Crtc1-flag and Crtc1b proteins in transfected cells suggested that Crtc1b expression is indeed lower than Crtc1 (data not shown), and hence complicates a “molar-to-molar” comparison of Crtc1 and Crtc1b coactivator activity in vitro.
4.3. Differential post-translational modification of Crtc1b isoforms

Crtc1b late isoform migrated faster than early isoform in denaturing polyacrylamide gel (Section 3.2; Fig. 3.5). This is surprising considering that Crtc1b late isoform (with additional Ex11_domain) is larger than Crtc1b early isoform (section 4.1). A probable reason for the observed size difference could be due to differential post-translational modifications, in which Ex11_domain could function as regulatory site (or direct target) for post-translational modifications (section 4.6). It is noteworthy to mention that Ex11_domain is rich in Ser/Thr residues, which could be potential phosphorylation sites (section 4.6), and important for modulation of protein-protein interaction (Nishi et al., 2011).

In addition to phosphorylation, O-glycosylation plays a role in regulating CRTC2 activity. It was shown that O-glycosylation by O-glycosyl transferase at Ser171 precludes SIK2/AMPK phosphorylation, and thus prevents sequestration of CRTC2 by 14-3-3 (Dentin et al., 2008). Multiple glycosylation of proteins are possible and Ex11_domain, which possesses several Pro and Ser residues are likely to be putative targets for glycosylation (Thanaka Christlet and Veluraja, 2001). However, such modification should increase Crtc1b late isoform and not the opposite.

Another possibility is that Ex11_domain stabilizes Crtc1b late isoform whereas the less stable early isoform is selectively polyubiquitinated. This could explain for the increased molecular weight and reduced protein level of early isoform as compared to late isoform (Fig. 3.5). Selective removal (or degradation) of maternal mRNAs and proteins is an integral part of maternal-to-zygotic transition process (DeRenzo and Seydoux, 2004; Tadros and Lipshitz, 2009). However, the mechanism for targeting maternal proteins for degradation is not fully understood.

Ex11_domain, which contain several Ser/Thr-Pro motif, could also be a target site for Pro isomerase Pin1 (section 4.6). Modulation of protein structure by Pin1 at the N-terminal of CRTC2 has been demonstrated to sequester the protein to the cytosol (Nakatsu et al., 2010). Pin1 changes protein conformation/structure by altering Pro configurations (Driver et al., 2014; Liou et al., 2011). In a denaturing SDS-PAGE, Pro-rich proteins have been known to produce a slower rate of gel migration,
suggesting that the different gel migration rate observed in Crtc1b isoforms are unlikely due to Pro isomerization.

4.4. Colocalization of meis1 and crtc1 expression

Cooperative interaction between the protein products of meis1 and crtc1 genes requires that they be expressed within the same tissue and at the same time. Their colocalization in the olfactory bulb, eye region and brain, among others, suggested that these domains are likely to be affected by the collaborative action of Meis1 and Crtc1 (section 3.3). Interestingly, creb1a is also expressed in these regions (http://zfin.org). Previously, we have shown that CREB can modulate MEIS1 activity and that both of them physically interact in cellulo (Looi, 2012), corroborating similar observation in MLL leukemia stem cell (Wang et al., 2010). Considering that Crtc1 acts as a coactivator for both CREB (Conkright et al., 2003) and MEIS (Goh et al., 2009; Looi, 2012; Wang et al., 2010), it would not be surprising if they cooperated during zebrafish development.

4.5. Distinct effect of tMO- and splMO-mediated knock down of crtc1

To knockdown crtc1, I have used both splice and translation MOs (section 3.4.1 and 3.4.2). Injection of crtc1 splMOs caused altered splicing and production of truncated proteins lacking the TAD region, which was expected to abolish Crtc1 coactivator activity (Fig 3.10C). In contrast, injection of tMO reduced Crtc1 protein by blocking translation (section 3.4.2.1; Fig 3.14). The resultant crtc1 tMO morphants displayed a consistent phenotype with a stereotypic curly-tail-down phenotype, whereas splMO morphants were more pleiotropic, albeit with overlapping defects with tMO morphants. These differences can be explained by comparing the mode of tMOs and splMOs action in zebrafish. For example, translation blockers act earlier to disrupt Crtc1 function by preventing the translation of maternally deposited transcripts and hence reduce Crtc1 protein level from earlier times and persisting to later developmental stages. On the other hand, splice blockers can only act later during zygotic expression, by which time maternal proteins have already been expressed. Secondly, reduced Crtc1 in tMO morphant is similar to Muller’s definition of hypomorph (in which gene
function is reduced but not completely lost; Muller, 1932), whereas disruptive splicing by splice MO can produce either loss- or gain-of-function (e.g. dominant negative) proteins. For Crtc1, loss of the TAD has been shown to confer a dominant negative property on the resulting protein. For example, human CRTC1 lacking the TAD was impaired in its ability to participate in the cAMP- and Ca\textsuperscript{2+}-mediated activation of CREB-dependent transcription in cortical neurons (Kovács et al., 2007). This is important because it could also means that dominant negative CRTC1 can suppress other CRTCs (e.g. CRTC1, CRTC2 and CRTC3) too, by competitive binding to MEIS and CREB proteins. This could be a reason for splMO morphants to display diverse phenotypic changes.

4.6. Partial functional redundancy of Crtc1 members

Knocking down crtc1b function with translation blocking morpholino did not produce any observable phenotypic changes (section 3.4.2; Appendix 3). This is probably due to compensatory activity by Crtc1, considering that they are closely related and share the same spatiotemporal expression. In contrast, Crtc1b was less effective in compensating for Crtc1 loss. Such a difference could be explained if we consider the dissimilarity in exon usage in Crtc1 and Crtc1b. Maternal protein for Crtc1b lacked Ex_11_domain (section 4.1), whereas Crtc1 retained the homologous amino acid sequence. This domain is not important for intrinsic co-activator activity (section 3.2), but, as mentioned above, this domain is close to the splicing regulatory domain of CRTCs (Amelio et al., 2009), and could be important for this aspect of Crtc1 function. Another possibility is that this domain could serve a regulatory role by post-translational modification, or protein-protein interaction. For example, Ser/Thr amino acid composition in Ex_11_domain is exceptionally high at ~28% (ProtParam, http://web.expasy.org/protparam/), which could be a potential target region of Ser/Thr kinases (predicted by Group based prediction system GPS tool) available at http://gps.biocuckoo.org/ (Xue et al., 2008). In addition, multiple sequence alignment of this domain with equivalent domains in Crtc1 and CRTC1 proteins from other organisms shows high occurrence of Ser-Pro motif (sequences marked in red box, Appendix 1). There are four Ser-Pro motifs in Ex_11_domain which are potential target sites of proline isomerase Pin1 that recognizes (Ser/Thr)-Pro motif. Previously, proline isomerization (that changes protein structure) of CRTC2 by Pin1 at N-terminus
site has been shown to sequester the protein to the cytosol (Nakatsu et al., 2010) and Ex_11_domain could provide additional target sites of Pin1 in CRTC1 family members. At later development (>72 hpf), although there was an increase in the amount of late Crtc1b, it was too late to compensate for crtc1 loss as crucial developmental processes have completed. In this regard, it would be interesting to determine if co-injection of mRNA for late Crtc1b can rescue crtc1 morphants.

4.7. **crtc1 morphants phenotype**

A striking feature of crtc1 tMO morphants is the curly-tail-down phenotype (section 3.4.2.2). In ENU-induced mutants, embryos with curly-tail-down phenotype and concomitant CNS defects are suspected to have defective midline structures (Brand et al., 1996). In addition, Creb deficiency in zebrafish causes abnormal somite and notochord morphology (Dworkin et al., 2007). Also, the CREB homolog Creb3l2 in X. laevis has been implicated in notochord development (Tanegashima et al., 2009). Taken together, these observations prompted us to study the midline structure of our morphants. We found that notochord maturation is hampered in crtc1 morphants as assessed by the persistent expression of shha and ihhb in 48 hpf notochord when it should be reduced (Fang, 2012; and discussion therein). Hh signalling from the notochord has been shown to differentially regulate epaxial (dorsal) and hypaxial (ventral) muscle development (Borycki et al., 1999; Martin et al., 2007). This is important in maintaining the balance of epaxial and hypaxial muscle mass (Martin et al., 2007). Considering that our morphants have higher shha and ihhb expression, myogenesis could be affected. However, we did not see any perturbation in the expression of myogenic genes (e.g. myf5 and myod1) or slow muscle smyhc1l (Fang, 2012).

Other phenotypic changes in crtc1 morphants are shorter body length, smaller eyes, and reduced brain size (see section 3.4.2.2). Shorter body length could be due to a defect in notochord function (Ellis et al., 2013; Odenthal et al., 1996), or convergent extension movement (Goudevenou et al., 2011). The latter is important for notochord function (Domingo and Keller, 1995; Glickman et al., 2003). Also, body growth has been shown to depend on insulin-like growth factor (IGF). Perturbation of this signalling pathway in zebrafish produces short embryos with smaller eyes, ear and
heart, and notably a curved body shape (Schlueter et al., 2006). The same pathway is also important for brain growth (Beck et al., 1995; Bondy et al., 2006). Interestingly, IGFs are expressed in the notochord and play an important role in peripheral tissue development (Escobar et al., 2011; Sang et al., 2008). Igf signalling plays a mitogenic role in brain development by influencing cell cycle regulators such as cyclin D1, D3 and E (Mairet-Coello et al., 2009) via the Ras/PI3K/p38 pathway to activate Creb (Pugazhenthi et al., 1999). Meis1 is shown to regulate the expression of cyclin D1 and c-myc (Bessa et al., 2008). Disrupting Creb and Meis functions in zebrafish have been shown to affect brain and eye development (Bessa et al., 2008; Dworkin et al., 2007). Collectively, these observations strongly hint at a similar role for CRTCs in this aspect.

In contrast, Crtc1 homozygous null mice show no obvious phenotype except for obesity (Altarejos et al., 2008; Breuillaud et al., 2009). Moreover, crtc1 deficiency in worms increased lifespan (Mair et al., 2011), whereas crtc homozygous null mutant flies displayed higher resistance to starvation with a concomitant increase in lipid and glycogen storage (Choi et al., 2011). Interestingly, deregulation of crtc expression in the fly eye caused ommatidial loss (Choi et al., 2011). All of the above suggested that CRTCs have a diverse role in development.

4.8. **Midline structure in crtc1 morphants**

The notochord is an important structure that provides early axial support. It is also involved in tail morphogenesis, as well as a source of patterning signals during embryonic development (Adams et al., 1990; Cunliffe and Ingham, 1999). Notochord development is dependent on multiple signalling pathways and interestingly, some of them share common effectors belonging to the CREB (ATF/CREM) family. For example, notochord development is regulated by Fgf3 (signal from neural floorplate) and non-canonical-Wnt/PCP (within the notochord), and both are mediated by a common JNK-ATF2 pathway (Ohkawara and Niehrs, 2011; Shi et al., 2009). In addition, CREB function in notochord development has been documented. For instance, *X. laevis creb3l* function is important during notochord formation and differentiation (Tanegashima et al., 2009). Moreover, several other biological processes important for notochord development are also regulated by CREB family
members, e.g. DV patterning (De Robertis and Kuroda, 2004; Keren et al., 2008), CE movements (Domingo and Keller, 1995; Glickman et al., 2003), gastrulation (Sundaram et al., 2003), and other morphogenetic movements (Zhou et al., 2007). In zebrafish, knocking down \textit{crtc1} significantly affected both early DV patterning (section 3.5.2.1) and convergence movement (3.5.2.3). In these morphants, mild defects in the establishment of left-right asymmetry were also observed (section 3.5.2.2), a phenotype consistent with a DV patterning defect (De Robertis and Kuroda, 2004).

Zebrafish with defective notochord often present abnormal body shape (Brand et al., 1996; Halpern et al., 1997). This is not surprising, considering that the notochord provides structural support to the embryo and as a source of patterning signals for peripheral tissues, e.g. muscle (Coutelle et al., 2001; Martin et al., 2007; Straface et al., 2009). Epaxial and hypaxial muscle development depends on signals from notochord, dorsal neural tube, and lateral plate mesoderm, among others, involving coordinated and combinatorial action of Hh, BMP and Wnt signals, which involves downstream effectors, e.g. PKA-CREB (Chen et al., 2005; Daury et al., 2001; Marcelle et al., 1997; Martin et al., 2007; Munsterberg et al., 1995). This is interesting, because CREB family members would therefore have important roles in the development of the notochord as well as muscle.

Neural crest migration behavior is dependent on notochord function, as Shh ligand has been shown to restrict (or inhibit) neural crest migration (Bronner-Fraser, 1993; Fu et al., 2004; Pettway et al., 1990; Testaz et al., 2001). Delayed reduction of Shh signal in notochord would therefore be expected to affect neural crest ventral migration. This was indeed observed as neural crest ventral migration was reduced in \textit{crtc1} morphants. Similarly, cranial neural crest migration to the terminal nerve was reduced (section 3.5.2.4).
4.9. Brain and eye development in crtc1 morphants

Zebrafish morphants were smaller with reduced brain and eye size. This is similar to zebrafish with disrupted Creb function, in which the larvae exhibit an abnormal body shape, reduced eye formation and microcephaly (Dworkin et al., 2007). Correspondingly, loss of Creb in homozygous null mice causes dwarfism and brain hypoplasia (Mantamadiotis et al., 2002; Mantamadiotis et al., 2006). Dwarfism in these mutants could be attributed to a hypothalamus-pituitary defect because Creb deficiency causes pituitary cell hypoplasia (Struthers et al., 1991) and this probably disrupts the production of pituitary growth hormone family members which are normally expressed within 24 hpf in zebrafish (Tian et al., 2010; Zhu et al., 2007). Interestingly, knocking down GH family members in zebrafish produces a phenotype reminiscent of that presented by Creb mutants and crtc1 morphants – i.e. short body length with smaller head and eye size (Zhu et al., 2007). As shown in section 3.5.2, the expression domain of several proneural genes was significantly reduced in morphants. Similarly, the expression domain of genes involved in retinogenesis was also diminished (section 3.5.1).

Reduced brain and eye size could be due to either decreased proliferation or increased apoptosis. In morphants, the proapoptotic gene cdkn1a expression was increased 4 fold (section 3.4.2.3; Fig 3.18). Robu et al. (2007) demonstrated that MOs can induce non-specific apoptosis in zebrafish. However, loss of Creb and Crem function in mice can also cause apoptosis and indiscriminate cell death in the nervous system (Mantamadiotis et al., 2002), probably via Creb-p53 interaction (Okoshi et al., 2011). This complicates the identification of bona fide apoptosis caused by gene knockdown using MO (Eisen and Smith, 2008; Gerety and Wilkinson, 2011). Although knocking down p53 in crtc1 morphant ameliorated some of the brain and eye phenotype, this was incomplete and much less effective than restoration of Crtc1 function (see rescue experiments in section 3.5.1 and 3.5.2). Apoptosis induced non-specifically by the morpholino should be refractory to rescue by crtc1 RNA, and so the robust rescue that I observe strongly validates a gene-specific phenotype. This indicates that apoptosis is likely just an accomplice rather than the culprit. However, future work is required to address this using alternative knockdown technology or by expressing a dominant negative Crtc1(Kovács et al., 2007).
Organ growth is regulated by cell proliferation. In the retina, proliferation and maintenance of retinal progenitor cells is governed by MEIS proteins through the control of cell cycle regulators (Bessa et al., 2002; Bessa et al., 2008; Heine et al., 2008; Pai et al., 1998; Pichaud and Casares, 2000). Reducing Meis1 expression in zebrafish delays G1/S transition and reduces cell proliferation resulting in reduced eyes (Bessa et al., 2008). In my crtc1 morphants, the expression domain of meis1 and meis2b in the eye field was reduced, suggesting that RPCs were reduced as well. Reduced amount of RPCs could probably be one reason for the subsequent reduced expression domain of retinal genes (section 3.5.1). Similarly, expression of the RPC marker olig2 (Hafler et al., 2012; Shibasaki et al., 2007) is contracted in morphants. Likewise, expression domains of genes involved in retinal differentiation were reduced too, such as isl1 and neurod4 (see section 3.5.1).

Neuron survival during development is dependent on proper axon pathfinding, synaptic connection and activity – all of which depend on correct patterning. Incorrect connections result in neuronal death, whereas proper connection and increased synaptic activity are important for survival (Barneda-Zahonero et al., 2012; Mao et al., 1999). Perturbation of this development could also contribute to the observed reduction in the eye and brain size of crtc1 morphants due to neuronal loss. In crtc1 morphants, meis1 expression was markedly reduced in olfactory bulb and retina. In comparison, Erickson et al. (2010) demonstrated that zebrafish meis1 is important for the patterning and connection of neurons in the retina to tectum. Although they did not address neuronal survival in their study, there was an indication of reduced neural mass suggesting that improper retinotectal connections had affected neuronal survival (or growth) – e.g. in their morphants, there was a 50% reduction in the tectal neuropil. Similarly, zebrafish with deficient pbx2 and pbx4 genes has smaller eyes and perturbed retinotectal connections (French et al., 2007). Once a connection is established, synaptic activity is required to promote neuronal growth and survival in a Creb-dependent manner (Barneda-Zahonero et al., 2012; Cho et al., 2013; Tan et al., 2012). Stimulation of Creb activity induces several genes important for neuronal growth and survival (Tan et al., 2012). Moreover, the persistent activation of Creb and Crtc1 proteins, which are involved in long term memory (Kovács et al., 2007; Zhou et al., 2006) is thought to promote cell growth and survival (Walton and Dragunow, 2000).
By the same token, chronic external stimuli (or stress) are pro-survival input for neurons via similar means (Sasaki et al., 2011). Collectively, all the above observations suggest that Meis1 and Creb activity in promoting neuronal survival/growth could converge at Crtc1.

4.10. CRTC1 in cell survival, proliferation and apoptosis

The bZIP family of transcription factor CREB/CREM/ATF and c-JUN/c-FOS are oncogenes. CREB and c-JUN/c-FOS have been shown to play a role in cell proliferation and cancer, by regulating the expression of genes involved in cell cycle progression (e.g. Ccnd1) and proapoptotic genes (e.g. p53 and p21) (Beier et al., 1999; Klein and Assoian, 2008; Schreiber et al., 1999). For example, c-Jun, which is a subunit of AP-1 transcription factor complex, has been shown to regulate apoptosis by antagonizing P53 activity in liver cancer mouse model (Eferl et al., 2003; Maeda and Karin, 2003). The deletion of c-Jun gene in mouse fibroblast cells leads to accumulation of P53, cyclin dependent kinase inhibitor Cdkn1, and Retinoblastoma 1 (Rb) with concomitant proliferation defect, whereas overexpression of c-Jun produces the opposite effect (Schreiber et al., 1999).

The cell proliferative and transformation property of AP-1 complex has been shown to be modulated by CRTC1, which associates with c-JUN and c-FOS at AP-1 target gene promoters in human cell lines (Canettieri et al., 2009). Similarly, CREB oncogenic (or cell proliferative) activity has also been shown to be dependent on CRTC1 activity (Gu et al., 2011; Johannessen et al., 2004; Katoh et al., 2006; Xiao et al., 2010). The role of MEIS1 in cell proliferation can be exemplified in leukemogenesis, and is dependent on the formation of HOX-PBX-MEIS complex, in which the transcription activity can be further enhanced by the incorporation of CRTC1 and CREB, to activate HOX-mediated leukemia-associated genes, e.g. FOS (Wang et al., 2010). Disrupting CREB and CRTC1 activity significantly reduced the cell proliferation rate of immortalized myeloid progenitors (Wang et al., 2010).

Surprisingly, CREB family protein has been shown to possess pro-apoptotic activity as well. CREB association with P53 has been demonstrated to attenuate the
expression of prosurvival \textit{MDM2} gene. This is achieved by reducing P53 occupancy in \textit{MDM2} promoter region, probably via sequestration of P53 into CREB-P53 protein complex, and thus prevents P53-activated \textit{MDM2} gene expression (Okoshi et al., 2011). CREB-P53 interaction requires CREB bZIP domain (Giebler et al., 2000; Okoshi et al., 2011). This protein domain is important for the dimerization of CREB family proteins (and c-JUN/c-FOS bZIP transcription factors) as well as association with other proteins, including coactivators such as CRTC family proteins (Johannessen et al., 2004). MDM2 is an E3 ubiquitin ligase that acts in a negative feedback mechanism to degrade its activator, P53, via the proteasomal degradation pathway to promote pro-survival and proliferative program. In contrast, the downregulation of MDM2 in response to glucose deprivation results in an increase in P53 expression and apoptosis (Okoshi et al., 2011).

How CREB switches its functional role from pro-apoptotic to survival is not known. One probable mechanism could be by competitive binding, whereby other coregulators can compete with P53 for binding to overlapping region of CREB bZIP. Such competition has been demonstrated for Endothelial Differentiation-Related Factor 1 (EDF1; aka MBF1) and K(Lysine) Acetyltransferase 7 (KAT7; homolog to fly Chameau) on the basic domain of bZIP of JunD protein. Under different environmental cues, EDF1 and MBF1 compete to interact with JunD to elicit different responses (Miller, 2009; Miotto and Struhl, 2006). Similarly, pro-apoptotic CREB-p53 protein complex could be modulated by pro-survival coregulators that compete for CREB binding and hence disrupt CREB-P53 complex formation. CREB-coregulators CRTC family proteins have been shown to be important for neuronal (Sasaki et al., 2011) and pancreatic (Jansson et al., 2008) cell survival. In neuronal cells, CRTC1 and CREB association has been shown to be important for promoting cell survival (Sasaki et al., 2011). All this information suggests that with the right signaling cue, CRTC1 is a likely candidate to compete with P53 for association with CREB. This would result in the activation of prosurvival \textit{MDM2} gene by P53, as well as other genes required for survival and proliferative signaling activated by CREB-CRTC1 (or in association MEIS-PBX) complex (Fig. 4.1). In the same vein, CREB-CRTC1-mediated gene activation has been shown to be important for long term memory formation and neuronal survival (section 4.7).
Alternatively, instead of competing with p53 for CREB binding, CRTC1 could synergize CREB-P53 activity to reduce MDM2 expression, and thus increase P53 protein level (Fig. 4.1). It is noteworthy to mention that P53 function is not exclusive to apoptosis but also important for promoting cell-cycle exit and cellular differentiation. Balancing the right P53 activity during embryogenesis is crucial for the regulation of cell proliferation and differentiation (Almog and Rotter, 1997; Molchadsky et al., 2010; Qin et al., 2007). For example, it has been noted that P53 activity is required for some cellular differentiation processes such as in hematopoiesis, spermatogenesis, adipogenesis, and muscle development (reviewed by Molchadsky et al., 2010). CREB has also been shown to be involved in adipogenesis (Hallenborg et al., 2012), osteogenesis (Kim et al., 2013), and hematopoiesis/vasculogenesis (Yamamizu et al., 2012). Furthermore, CREB, P53, CBP, and KLF4 have been shown to act in a combinatorial manner to activate bradykinin receptor B2 (BDKRB2) gene, which is expressed during terminal nephron differentiation (Saifudeen et al., 2005). In this alternative pathway, CRTC1 would be expected to increase P53 protein level and synergizes with CREB-P53 transactivation activity to promote cell-cycle exit and cellular differentiation.

In the crtc1 morphants, brain and eye sizes were significantly smaller than uninjected controls. Moreover, cells expressing the markers for neural precursor cells and differentiated neurons were markedly reduced in the brain and eye regions (section 3.5.1. and 3.5.2). All these results suggest that proliferation or differentiation process could be perturbed in crtc1 morphants. Another possibility for the reduced number of progenitor or differentiated cells in these regions could also be due to cell death/apoptosis. The preliminary ISH result indicated that the expression of p53 and cyclin D1 are both increased in crtc1 morphants, suggesting that both processes are likely to be perturbed (data not shown). Interestingly, ccnd1 showed persistent expression in the brain and spinal cord of 48 hpf morphants when it was supposed to be downregulated. This could mean a perturbed neuronal differentiation in crtc1 morphants, especially considering that the downregulation of ccnd1 expression is required for cell differentiation (He et al., 2011; Nishi et al., 2009).
Figure 4.1 CRTC1 role in cell proliferation, survival, apoptosis, and differentiation
4.11. CRTC1 in other embryonic processes

Considering that CRTC1 proteins are coregulators for transcription factors involved in a multitude of signaling pathways (under Pathways and Interactions in http://www.genecards.org), it is very likely that CRTC1 participates in other embryonic processes as well. For example, the role of CRTC1 in embryonic patterning could be linked to its association with MEIS1 and CREB. MEIS1 is important for embryonic patterning and has been shown to be involved in eye patterning and retinotectal pathfinding (Erickson et al., 2010). Similarly, a p38-CREB pathway has been observed to play a role in DV patterning of mesoderm during early gastrulation downstream of maternal Wnt/β-catenin signal and organizer genes siamois and goosecoid in frog (Keren et al., 2008). In crtc1 morphants, DV patterning is shown to be deviated from control morphants (section 3.5.2.1). It is interesting to note that perturbed DV patterning that causes either ventralized or dorsalized phenotype usually affect notochord development in zebrafish (Kishimoto et al., 1997; Mullins et al., 1996).

The Wnt/Planar-Cell-Polarity (Wnt/PCP) pathway is important for morphogenetic movement, which is important for convergent extension movement (Heisenberg et al., 2000), cell adhesion (Kraft et al., 2012), cardiac outflow tract formation, and probably in other normal cell migration processes (Zhou et al., 2007). The JNK-ATF/CREB pathway, downstream of Wnt11/PCP, has been shown to be required for the activation of the TGFβ2 gene important for outflow tract morphogenesis (Zhou et al., 2007). On a side note, maternal CREB has been shown to activate the X. laevis brachyury (xbra) gene required for gastrulation movement (Conlon et al., 1996; Sundaram et al., 2003). If indeed CRTC1 is involved in morphogenetic movements, it would likely be acting along the JNK-ATF/CREB pathway. In crtc1 morphants, convergence movement was perturbed, while extension was not affected (section 3.5.2.3).
CHAPTER 5 : CONCLUSION & FUTURE DIRECTIONS

The use of zebrafish as a model to study Crtc1 function provided a glimpse into the possible developmental processes involving Meis and Crtc1 proteins. Similar to Meis1/Pbx-deficient zebrafish, our morphants displayed smaller eyes and brain suggesting a probable disruption in the organization of the retinotectal map. Considering that proper synaptic connections are important for neuronal survival/growth, and that Creb is also involved in this process, it is probable that Crtc1 is involved as well.

We have also shown that the abnormal body curvature in our morphants could be due to defective notochord function, and this was corroborated by ISH analysis indicating a perturbed Hh signalling. Notochord development is the result of a culmination of several developmental processes encompassing early DV patterning and CE movement during gastrulation and involving several signaling pathways such as FGF and Wnt. All these processes have been linked to the activity of CREB, and thereby implicating an involvement of Crtcs as well.

The role of Crtc1 in development is extensive thanks to its association with several transcription factors, namely MEIS and bZIP family members. A good tissue in which to focus future work would be the retina. Considering that Creb and Meis are important in eye development (and retinotectal connections), the retina would be an ideal in vivo model to test the cooperation of Creb, Meis and Crtc1. Moreover, we have shown the interaction and cooperative activity of CREB and MEIS proteins in vitro (Looi, 2012) – corroborated by the work of Wang et al. (2010) – and extending our studies to the retina would allow us to validate these findings in a physiological setting.

As previously mentioned, perturbation of Crtc1 function could affect retinal patterning, axon pathfinding, maintenance and proliferation of RPCs, retinal differentiation, neuronal activity and cell survival or death. These effects share a common mechanism involving apoptosis. In order to address whether cell death observed in our morphants is due specifically to crtc1 knockdown, another approach is
required. Recently, we constructed a dominant negative Crtc1 according to Kovács et al (2007). However, in order to produce a dominant negative effect, overexpression of this construct is required and this could be toxic to the zebrafish embryo. To circumvent this complication, we have generated a similar construct but fused to the zebrafish Hes6 WRPW repressor domain (240-334), which is known to interact with the Groucho/TLE repressor complex (Fisher et al., 1996; Grbavec and Stifani, 1996). I observed that Crtc1-WRPW is able to silence CREB activity in a luciferase reporter assay, even in the presence of exogenous Crtc1 (data not shown) suggesting a potent dominant negative Crtc1 candidate. A caveat to this approach is that, contrary to the use of morpholinos, dominant negative Crtc1 would affect the function of all CRTCs and not just Crtc1.

There are several questions that remain unanswered:

(1) Are Crtc1 and Meis1 co-expressed in the same cell? I have used ISH to establish where and when meis1 and crtc1 are co-expressed in the zebrafish embryo. To determine whether protein products of these genes are colocalized in the same cell, several methods can be employed such as immunofluorescent detection or the proximity ligation assay (PLA). Testing of commercially available antibodies against Crtc1 will be required.

(2) Are retinotectal connections perturbed? To address this question, several methods can be used such as anterograde tracing of RGC, immunochemical detection of neurons (e.g. anti-acetylated tubulin), transgenic reporter lines [e.g. isl2b:GFP and brn3c:GFP zebrafish reporter lines, both of which specifically delineate RGCs (Pittman et al., 2008; Xiao et al., 2005)], among others.

(3) Is there perturbation of eye patterning in crtc1 morphants? I have shown that meis2b expression was not overtly affected in the head region, whereas meis1 expression was severely reduced in the olfactory bulb and retina in 48 hpf morphants. Previously, targeted inactivation of meis1 in zebrafish by morpholino has been shown to perturb retinal patterning at the DV and nasal-temporal (NT) axes by altering the expression domains of ephrin ligand (efn) and ephrin receptor (eph) genes (e.g. dorsal retina expresses efnb and ventral retina expresses ephb; similarly nasal and ventral
retina expresses efnα and epha, respectively). Several signaling pathways important for the correct establishment of the “eph-efn code” (or eph-ephrin code) were also affected, e.g. loss of dorsal tbx5 expression (Bmp target gene), and downregulation of temporal expressed foxd1 (Erickson et al., 2010). To determine if crtc1 morphants have similar eye patterning defects, several of the aforementioned genes can be analysed by ISH.

(4) Is retinal lamination affected? Smaller eyes could be due to perturbed retinal differentiation (or lamination). To study whether retinal lamination is affected, several methods can be used such as histological examination of retinal section (e.g. Haematoxylin and Eosin staining), immunohistochemical detection of retinal cell types (e.g. Ab1-Pax6a antibody for detection of both RGC and amacrine cell), and ISH detection [e.g. RGC marker cxcr4b, amacrine marker pou3f3b, and others as described by Pujic et al. (2006)].

(5) Once the above is established, the next question is to ask whether there is cooperative action between Meis1, Creb and Crtc1. This can be addressed by combinatorial knockdown of these proteins using MO technology or by recently available knockout approaches. For example, in order to determine whether there is genetic interaction between Meis1, Creb1 and Crtc1, titration of each of the MOs to concentrations that produce aphenotypic/asymptomatic morphants is required. These aphenotypic morphants are assumed to have “hypomorphic” levels of gene function due reduced level of morpholino knockdown after titration. Manipulation of morpholino levels to achieve hypomorphism has been reported (reviewed in Gerety and Wilkinson, 2011). Next, according to the genetic enhancer (or genetic interaction) model, the summed effect of two hypomorphic genes is expected to produce a phenotype if these genes interact (Herman and Yochem, 2005). This can be accomplished by co-injection of reduced concentrations of morpholinos targeting either meis1+crtc1, meis1+creb1, creb1+meis1 or meis1+creb1+crtc1.

(6) Last but not least, a gene expression profile database of Crtc1, Meis1 and Creb function is needed as reference for the identification of common pathways regulated by these proteins and for studying the mechanism of such gene regulation. We have collaborated with the group of S. Mathavan (GIS, Singapore) to use microarrays to characterize alterations to the transcriptome of zebrafish embryos lacking crtc1.
functions (data not shown). Of 111 genes downregulated by 3-fold in 48 hpf, Panther classification (http://www.pantherdb.org) places 31 genes under developmental processes (GO: 0032502). Out of these 31 genes, 13 genes are expressed in the eye region (e.g. irx6a, bhlhe23, and tfap2d, rorb, lhx1a, among others) and several of these genes have been implicated in the specification of retinal cell types, e.g. irx6a in regulating retinal interneuron subtype identity in chick (Star et al., 2012), bhlhe23 function in the specification of amacrine and cone bipolar subtypes in mouse (Feng et al., 2006) and rorb in rod photoreceptor development (Jia et al., 2009). The rorb gene is also important in the regulation of retinal progenitor proliferation (Chow et al., 1998). Next, lhx1a which is found in the proximal region of nascent optic cup is important in optic cup development, as overexpression of this gene in the chick outer pigmented retina (or retinal pigmented epithelium) induces a second retina (Kawae et al., 2012).
1. Multiple amino acid sequence alignment of zebrafish Crtc1 and Crtc1b with CRTC1 subfamily members from other organisms
Appendix 1  Multiple amino acid sequence alignment of Crtc1 and Crtc1b with CRTC1 family members of other organisms. Identical residue are denoted as asterisks (*), conserved substitutions denoted as colon (:), and semi-conserved substitutions denoted as dot (.). The long gaps caused by alternative splicing greatly affected the homology score and made such regions to have misleadingly “low conservation”. The red boxes marks the exon 11 of crtc1b gene. Zebrafish Crtc1, early and late isoforms of Crtc1b are denoted as zCrtc1, zCrtc1b_early and zCrtc1b_late. The highly conserved Ser-Pro motifs in CRTC1 family members are shown as underlined *. Blue box marks the splicing domain mentioned in work by Amelio et al. (2009).
2. Multiple amino acid sequence alignment showing exon 11 of Crtc1b aligned with comparable region of other CRTCs (e.g. Crtc2 and Crtc3) of human and mouse.

<table>
<thead>
<tr>
<th></th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCRTC1</td>
<td>436 -----------------------IDIASAPALQQYRTSAGSPANQSPTSPVSNQGFS-----PGSSP</td>
</tr>
<tr>
<td>mCrtc1</td>
<td>433 -----------------------IDATSAPALQQYRTSAGSPANQSPTSPVSNQGFS-----PGSSP</td>
</tr>
<tr>
<td>zCrtc1b_late</td>
<td>390 -----------------------IDINTAASLQQYRCRVGSSANQSPTSPVSNQGFS-----PGGSP</td>
</tr>
<tr>
<td>zCRTC1_Xp_003199473.1</td>
<td>387 -----------------------SPTTTGTAASLQQYRTSAGSPANQSPTSPVSNQGFS-----PGGSP</td>
</tr>
<tr>
<td>zCRTC3</td>
<td>422 -----------------------PAAMHLGIGNSQGLSSSLSNPSIQASLTNSQLHSSLTSQSD</td>
</tr>
<tr>
<td>mCrtc3</td>
<td>433 -----------------------PITDRAAASLQQYRTSAGSPANQSPTSPVSNQGFS-----PGGSP</td>
</tr>
<tr>
<td>hCrtc3</td>
<td>487 -----------------------PYSSPSLVLPTQPHTPKSLQQPGLPSQSCSVQSS-----GGQPP</td>
</tr>
<tr>
<td>mCrtc3</td>
<td>488 -----------------------PYSPPSLVLPTQPHTPKSLQQPGLPSQACLVQPS-----GGQPP</td>
</tr>
<tr>
<td>zCRTC2</td>
<td>476 SHHIRPAPPLSHQPLRQYPFSPQPELELQWIEHQQHHNQGATPVHKRTHA</td>
</tr>
</tbody>
</table>

Appendix 2: Multiple amino acid sequence alignment of exon 11 of Crtc1b aligned with comparable region in other families of CRTCs of human and mouse. Red box marks the exon 11 region of Crtc1b.
Appendix 3  In a dose-response experiment to determine sufficient dosage to elicit a phenotypic change while minimizing non-specific side-effects, embryos were injected with (B) 2 ng, (C) 4 ng, (D) 6 ng, and (E) 8 ng of crtc1b tMO1. These were compared to embryos injected with 6 ng of control MO. Injection of up to 8 ng of tMO1 did not cause any observable change in morphants. Red scale bar = 500 µm.
4. List of DNA constructs used in the study

pST Meis2b

Vector backbone: pST
Insert sequence: EcoRI-meis2b-XbaI

```
gaattcagctcggtaaccatGGGATGAGATGGATGAGATGGGACATGGGACATTACATGGGAACACGCCTCCCGCTCCCAACCCATATAAAGCCTGTTTGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATGTTTGGATGTTTGGATCATTAG
TTTTAGACCTTTGGCATTTTCTGACTACAGTGTTTGGATG TT
```
pST zGsc

Vector backbone: pST
Insert sequence: BamHI-gsc-XhoI

```
ggatccactaaggggccgcaagctgtgctgattcaagctgatGAGACGACACCGAACCATTTTCACCGACGAGCAGCTGGAGGCACTGGAAAACCTTTTTCAAGAAACGAAATACCCTGACGTCGGCACAAGAGAACAAGGTGCACCTACGTGAAGAGAAGGTAGAGGTTTGGTTCAAAAACAGACGAGCAAAAACGGAGAAGACAGAAAAGGTCGTCGTCAGAGGAATCAGAAAACTCACAGAAATGGAACAAATCCACGAAAACAACCTCAGAGAAAATTGAGGAGGGCAAAAGCGACGTGGATTCTGACAGCTGATATAAAGAACACGAGAACGGGAAATCTTGCAAAATATAATTCGGACTTTATTGTACATATTGTAAAGTATGTGAGTTGGAGTAAATAGTGTTGTCTTGCTGTTGACATGTATAGTAAATGTTACCTTTAACAATGATATAGTTATTTATATTAATTTAATTATTCAACAGCAAAAATACGTTGACATTCTAGCCAATTGATATATTTACCTGCTGATATCTGCAAACTATTTAAAAAGCTTTTGGCTGTAAGATGTAACCTAAATCTGATGCTGTTGGAATGGGCTGTAGCTGAA
```

GGACAAAGCTCTGAGATACGATGGCAGGACGTACATTCGGACATTAATTAATTTAAAGTTTAATGTCGCCTGAAAACGAACAAATATTAAAGTAATATCTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAActcgag
p4T cyp26a1

Vector backbone: pCR4 TOPO (Invitrogen)
Insert sequence: PmeI-cyp26a1-NotI

gttttaaaccg...
pSP zCRTC1-flag

Vector backbone: pSP64 (Promega, cat. no. P1241)
Insert sequence: HindIII-crtc1-FLAG-XbaI

Highlighted yellow is the FLAG epitope nucleotide sequence
pSP RzCRTC1-flag

Vector backbone: pSP64
Insert sequence: HindIII-tMO-resistant crtc1-XbaI

Highlighted yellow is the FLAG epitope nucleotide sequence.
pcDNA3.1 Crtc1

Vector backbone: pcDNA3.1(+) (Invitrogen)
Insert sequence: XbaI-crtc1-XbaI

tctagacaacgcgtcttcacgcacagccttcgttgttttgaggaagacgagatATGGCGTCCTCAAACAATCCGCGAAAATTCAGCGAGAAAATCGCCCTGCACAACCAGAAGCAGGCGGAGGAGACCGCGGCGTTTGAGGAGGTCATGAAAGACCTGAGCATCACGCGAGCTGCACGGCTGCAGTTACAGAAGACCCAGTATTTGCAA
CTAGGCCAGAATCAGCGTCATGCTATGGAAGGTCAGCTCAGCGACTAAATGGCAATTAGGAAATAGCAACACTGACCTGACCTCAGCTCAGCTACCAGAGCGGTGTTGAAATCAACGTTAGTGCAGACAGCTGCCCTTATGGCTCGGTATATCTGTCACCGCCTCCTGACACAAGCTTGCGGGA
TCTAGAAATGGAAAGATGATTCCAAGACATCTCTGCTCACAGATTCCTGTGATGTTCCTGGCATT
AACATATTTCCCTCCTCTCCGAGTAAAGAATGATTGCTCTTGAGACAGGTCTCACAGCACAGGCGGCTCTTTACCTGATCTGACAAACATCCAGTTCCCTCCTCCCCTCCCGACCCCACTGGATCCGGACGACCCTATTACCTTTCCTACATCCAGTTCCAGCAGTACCAGCAACCTGACCACCAATCTGACCCACCTGGGGATCAGTGCTGCCAGTCACGTTCCTCCCTCCCCTCCAGCCCCACACACACGATCAGGACGGGGGAGTCTCCTCCCAGTCTCTCCAAAGACCTCACCAGCTCATTGGCCGGCGTTGGTGAGGTCAGCTTTGATGCGGACTCGCAGTTCCCATTGGACGAACTGAAGATCGACCCCTTGACACTGGACGGCACTGCACATGCTAAACGACCCAGACATGGTTCTCGCCGACCCGGCCACCGAGACACTTTCCGCATGGATCGACTGTGAacctttgaatgcaattttaagcgagacatttctgctgcatggcctctctgttcaccatctggtcagtggtctaga
pcDNA3.1 Crtc1-flag

Vector backbone: pcDNA3.1
Insert sequence: HindIII-crtc1-FLAG-XbaI

Highlighted cyan is the 5'UTR. Highlighted yellow is the DNA sequence coding for flag epitope. DNA sequence in small letter is from vector backbone.
pcDNA3.1 early Crtc1b

Vector backbone: pcDNA3.1(+)  
Insert sequence: XbaI-early crtc1b-xbaI

Highlighted cyan is 5'UTR. Highlighted yellow is the 88 bases of 3'UTR of zebrafish crtc1b mRNA after the stop codon.
pcDNA3.1 late Crtc1b

Vector backbone: pcDNA3.1 (+)

Insert sequence: XbaI-late crtc1b-XbaI

tctaga

Highlighted cyan is the 5'UTR. Highlighted yellow is the 88 bases of 3'UTR of zebrafish crtc1b mRNA after the stop codon.
pCS2+ Crtc1-flag

Vector backbone: pCS2+
Insert sequence: EcoRI-crtc1-FLAG-Xbal

gaattcgcctccctaacgctctgtggttttggagaagaagctggaagATGGCGTCCTCAAACAATCCGGCAGAAATTCAGCGAGAAAATCGCCCTGCACAACCAGAAGCAGGCGGAGGAGACCGCGGCGTTTGAGGAGGTCATGAAAGACCTGAGCATCAGCGAGCTGCACGGCTGCAGTTACAGAAGACCCAGTATTTGCAACTAGGCCAGTGACAGCTCTTTTATGGCTCGGTATATCTGTCACCGCCTCCTGACACAAGTTGGCGGAGAACAAACTCTGACTCAGCTCTACACCAGAGCGCTGTGAATCCAGCTCCACAGGACTCTTTTGTTGGTGGATCTCAGGACTGCAGTCAAAAGGCGTAAAGACATTGCTGTTGCTCACCCCTCCGGACACAGAAGACGCTGAATCAGAACATGGAGAAAGATGATTCCAAGACATCTCTGCTCACAGATTCCTGTGATGTTCCTGGCATTAACATATTTCCTCTCTCCCTCCCTCCCTCCAGCCCAGCACACAGGCTCCGCCCCCTCACCCAGCCTCAGCCAACGCAAACCACACTGCCGTCTGCTGCCTCTCCTCAGATACCCACATTGCCGACACAGTGATGAGTGATCAGTCTGTTACTGGAGCAGCAGTTGTCTCAGTACTCTGCGTGCTGAGTTTACTGAACGACCTGCAGAAGCAAGCACACCTTCCCTCAGAACATCCGCTCTACAGGCTCCCGCGCCCTCCCTACCCGCGGACCGCCGCCCCCTCAGGCGACACACACACATGACGAGAAGAGCTCGGACAGACCTCGCGATGGGAGTCTCCTCCCAGTCTCTCCAAAGACCTCACCAGCTCATTGGCCGGCGTTGGTGACGTCAGCTTTGATGCGGACTCGCAGTTCCCATTGGACGAACTGAAGATCGACCCCTTGACACTGGACGGACTGCACATGCTAAACGACCCAGACATGGTTCTCGCCGACCCGGCCACCGAGCACTTTCCGCATGGATCGACTGGA

TATCGACTACAAGGATGATGACGACAAGGCGCTGTAatctaga
pCS2+ vector
pST vector

Vector backbone: pUC19
Insert sequence: SP6-MCS-T7

CATACC\textcolor{cyan}{ATTAAGGTGACACTATAGA}ATACCTCAAGCTATGCAATCAAGCTTTGTAACGGCTGGCCTCGGATCC
\textcolor{yellow}{TAGTAAACGCGCCGCCAGTGCGGAAATTCTCGAGATATCCATACACTGCGCCGCTTCGACGATCATGCATCTAGAGGCGGCCCATTCCCGCCCTATGCGG
\textcolor{cyan}{CCCTATAGTGAGTGATATTCTACATGCAAGCTATGCAATCAAGCTTTGTAACGGCTGGCCTCGGATCC

Highlighted cyan corresponds to SP6 site, while highlighted yellow corresponds to T7 site.
5. Sense control for WISH of *crtc1* and *crtc1b* expression in zebrafish embryos

Appendix 5a Whole mount in situ hybridization of *crtc1* expression during zebrafish embryo development as assessed by DIG-labelled sense RNA probe. The use of sense probes in conjunction with antisense RNA probes (see Fig. 3.1.) is to reduce the probability that the signal observed in antisense probe is due to background staining. Developmental stages of samples are (A) 1-cell (0 hpf), (B) 16-cells (1.5 hpf), (C) 64-cells (2 hpf), (D) sphere (4 hpf), (E) germ ring (5.7 hpf), (F) bud (10 hpf), (G) 6 somites (12 hpf), (H) 24 hpf, and (I) 48 hpf.
Appendix 5b Whole mount in situ hybridization of crtc1b expression during zebrafish embryo development as assessed by DIG-labelled sense RNA probe in comparison to antisense RNA probe (see Fig. 5.2.). Developmental stages of embryos are (A) 16-cells (1.5 hpf), (B) sphere (4 hpf), (C) germ ring (5.7 hpf), (D) bud (10 hpf), (E) 6 somites (12 hpf), (F) 24 hpf, (G) 30 hpf, and (H) 48 hpf.
6. Dynamic expression of 18S rRNA during zebrafish development

Appendix 6  Quantitative RT-PCR result indicates the dynamic changes in 18S rRNA expression during embryonic development in zebrafish at 1-cell, 16-cells, sphere, germ ring, 24 hpf, 48 hpf, and 72 hpf stages. NTC = no template control. The error bar indicates four technical replicates (n = 4) from two independent experiments.
7. *p53* and *delta113 p53* expression in controls and *crtc1* morphants

Appendix 7  Quantitative RT-PCR result showing the expression of *p53* and *delta113 p53* (implicated in MO-mediated unspecific apoptosis) in 24 hpf embryos of uninjected, control MO injected (con), *crtc1* tMO injected (c1_1), control MO and *p53* tMO co-injected (con+p53), and *crtc1* tMO and *p53* tMO co-injected (c1_1+p53) embryos. NTC = no template control. The error bar represents three technical replicates of 50 embryos per RNA sample.
8. Gene and protein nomenclature guideline

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene symbol</th>
<th>Protein symbol</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Homo sapiens</em></td>
<td>SHH</td>
<td>SHH</td>
<td>HUGO Guideline (<a href="http://www.genenames.org/hgnc-guidelines">http://www.genenames.org/hgnc-guidelines</a>; accessed on 13 Aug 2014)</td>
</tr>
<tr>
<td><em>Gallus gallus</em> (chick)</td>
<td>shh</td>
<td>SHH</td>
<td>(Burt et al., 2009)</td>
</tr>
<tr>
<td><em>Xenopus laevis</em> or <em>Xenopus tropicalis</em></td>
<td>shh</td>
<td>Shh</td>
<td>Xenbase Guideline (<a href="http://www.xenbase.org/gene/static/geneNomenclature.jsp">http://www.xenbase.org/gene/static/geneNomenclature.jsp</a>; accessed on 13 Aug 2014)</td>
</tr>
</tbody>
</table>
REFERENCES


Berthelsen, J., Kilstrup-Nielsen, C., Blasi, F., Mavilio, F., Zappavigna, V., 1999. The subcellular localization of PBX1 and EXD proteins depends on nuclear import and export signals and is modulated by association with PREP1 and HTH. Genes & development 13, 946-953.


165


Du, K., Montminy, M., 1998. CREB is a regulatory target for the protein kinase Akt/PKB. Journal of Biological Chemistry 273, 32377-32379.


169


Fang, X.T., 2012. The role of crtc genes in the development of the zebrafish embryo. Nanyang Technological University, Singapore.


Ferretti, E., Schulz, H., Talarico, D., Blasi, F., Berthelsen, J., 1999. The PBX-regulating protein PREP1 is present in different PBX-complexed forms in mouse. Mech Dev 83, 53-64.


signaling axis promotes esophageal cancer cell migration and invasion. Oncogene 31, 469-479.


Horan, G.S., Ramirez-Solis, R., Featherstone, M.S., Wolgemuth, D.J., Bradley, A., Behringer, R.R., 1995b. Compound mutants for the paralogous hoxa-4, hoxb-4, and hoxd-4 genes show more complete homeotic transformations and a dose-dependent increase in the number of vertebrae transformed. Genes Dev 9, 1667-1677.


Looi, Y., 2012. Elucidation of the physical and functional interactions between the MEIS1A and the CREB and CRTC transcription factors, School of Biological Sciences. Nanyang Technological University, Singapore.


causes anterior pituitary hypoplasia and dwarfism in mice. Molecular Endocrinology 20, 204-211.


localization of another homeoprotein, extradenticle, and suppresses eye development in Drosophila. Genes & development 12, 435-446.


Stevens, K.E., Mann, R.S., 2007. A balance between two nuclear localization sequences and a nuclear export sequence governs extradenticle subcellular localization. Genetics 175, 1625-1636.


