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<td>Hensel, Katrin</td>
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Wnt signalling in the Hydrozoan

*Hydractinia echinata*

Author: Katrin Hensel
Supervisor: Dr. Uri Frank

Discipline: Zoology

School of Natural Sciences and
Regenerative Medicine Institute (REMEDI)
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Galway, Ireland

Thesis submission September 2013
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table of Contents</td>
<td>3</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>7</td>
</tr>
<tr>
<td>Summary of Content</td>
<td>8</td>
</tr>
<tr>
<td>Declaration of contribution</td>
<td>9</td>
</tr>
<tr>
<td>List of Abbreviation</td>
<td>11</td>
</tr>
<tr>
<td>List of Figures</td>
<td>14</td>
</tr>
<tr>
<td>List of Tables</td>
<td>20</td>
</tr>
<tr>
<td><strong>1 Chapter One – General Introduction</strong></td>
<td>21</td>
</tr>
<tr>
<td>1.1 Wnt signalling</td>
<td>22</td>
</tr>
<tr>
<td>1.2 Examples of expression patterns and functions of different Wnt ligands</td>
<td>29</td>
</tr>
<tr>
<td>1.3 <em>Hydractinia echinata</em> as a model system</td>
<td>37</td>
</tr>
<tr>
<td>1.4 Aims of the research</td>
<td>45</td>
</tr>
<tr>
<td><strong>2 Chapter Two - Material and Methods</strong></td>
<td>47</td>
</tr>
<tr>
<td>2.1 Animal culture</td>
<td>47</td>
</tr>
<tr>
<td>2.1.1 Culturing</td>
<td>47</td>
</tr>
<tr>
<td>2.1.2 Metamorphosis</td>
<td>47</td>
</tr>
<tr>
<td>2.2 Protocols</td>
<td>48</td>
</tr>
<tr>
<td>2.2.1 Total RNA extraction</td>
<td>48</td>
</tr>
<tr>
<td>2.2.1.1 Small sample amounts</td>
<td>48</td>
</tr>
<tr>
<td>2.2.1.2 Big sample amounts</td>
<td>49</td>
</tr>
<tr>
<td>2.2.2 DNase digestion of total RNA</td>
<td>50</td>
</tr>
<tr>
<td>2.2.3 Formaldehyde denatured 1.5 % gel for RNA analysis</td>
<td>51</td>
</tr>
<tr>
<td>2.2.4 cDNA synthesis</td>
<td>51</td>
</tr>
<tr>
<td>2.2.4.1 RT cDNA was transcribed according to the protocol for Omniscript Reverse transcriptase</td>
<td>51</td>
</tr>
<tr>
<td>2.2.4.2 RACE cDNA</td>
<td>52</td>
</tr>
<tr>
<td>2.2.5 PCR</td>
<td>53</td>
</tr>
<tr>
<td>2.2.5.1 PCR using GoTaq® DNA polymerase</td>
<td>53</td>
</tr>
<tr>
<td>2.2.5.2 PCR using Phusion High Fidelity™ DNA Polymerase</td>
<td>54</td>
</tr>
<tr>
<td>2.2.5.3 Cloning of PCR products with phosphorylated primers</td>
<td>55</td>
</tr>
<tr>
<td>2.2.6 Sequencing</td>
<td>56</td>
</tr>
<tr>
<td>2.2.7 Gel extraction</td>
<td>57</td>
</tr>
</tbody>
</table>
# Table of Contents

2.2.8 DNA clean up with phenol/ chloroform and precipitation .......................... 58  
2.2.9 DNA ligation ......................................................................................... 58  
2.2.10 Cloning ................................................................................................. 59  
2.2.11 Plasmid extraction from bacteria .......................................................... 59  
2.2.12 RNA probe synthesis for *in situ* ......................................................... 60  
2.2.13 Whole mount *in situ* hybridisation ..................................................... 62  
2.2.14 EdU staining .......................................................................................... 64  
2.2.15 May-Grünwald/ Giemsa staining .......................................................... 65  
2.3 Pharmacological Wnt deregulation ............................................................. 66  
2.3.1 Wnt activation ......................................................................................... 66  
2.3.2 Wnt inhibition ........................................................................................ 66  
2.4 Flow cytometry ........................................................................................... 68  
2.4.1 Dissociation protocol and sample preparation ........................................ 68  
2.4.2 Sample run/ parameters ......................................................................... 68  
2.5 Oligonucleotides used in the study .............................................................. 70  
2.6 RNA probes used for expression analysis .................................................. 72  
3 Chapter Three – Gene cloning and sequence analysis ............................... 75  
3.1 Introduction ............................................................................................... 75  
3.2 Experimental approach .............................................................................. 77  
3.2.1 Gene amplification by RACE PCR ......................................................... 78  
3.2.2 Phylogenetic analysis ............................................................................. 78  
3.3 Wnt1 .......................................................................................................... 80  
3.4 Wnt2 .......................................................................................................... 83  
3.5 Wnt5B ....................................................................................................... 86  
3.6 Wnt7 .......................................................................................................... 89  
3.7 Wnt8 .......................................................................................................... 92  
3.8 Wnt9/10 ................................................................................................... 95  
3.9 Wnt11A .................................................................................................... 98  
3.10 Wnt11B .................................................................................................. 101  
3.11 Wnt16 ..................................................................................................... 105  
3.12 Summary .................................................................................................. 108  
4 Chapter Four – Gene expression analysis ..................................................... 109  
4.1 Introduction ............................................................................................... 109  
4.2 Experimental approach ............................................................................. 112
Table of Contents

4.3 Expression pattern during embryonic development ........................................... 113
4.4 Expression patterns at the larva stage ............................................................ 118
4.5 Expression patterns after metamorphosis induction ......................................... 122
4.6 Expression patterns in stolons ........................................................................ 125
4.7 Expression patterns in adult feeding polyps ...................................................... 129
4.8 Expression patterns in sexual polyps ............................................................... 136
4.9 Summary ........................................................................................................ 138

5 Chapter Five – Gene expression after Wnt deregulation .................................. 142
5.1 Introduction ..................................................................................................... 142
5.2 Experimental approach ................................................................................... 145
5.3 Wnt activation ................................................................................................. 147
5.3.1 EdU staining after Wnt activation .............................................................. 147
5.3.2 Expression pattern after Wnt activation .................................................... 150
5.4 Wnt inhibition ................................................................................................ 162
5.5 Summary ........................................................................................................ 163

6 Chapter Six – Flow cytometry as an analytical method for Hydractinia cell biology ................................................................. 165
6.1 Introduction ..................................................................................................... 165
6.2 Experimental approach ................................................................................... 168
6.3 Flow cytometry of Hydractinia echinata ......................................................... 170
6.4 FACS of wild type and Pln transgenic animals ................................................. 177
6.5 Flow cytometry after Wnt activation ............................................................... 184
6.6 Summary ........................................................................................................ 187

7 Discussion ........................................................................................................... 188
7.1 Identification of new Wnt ligands ................................................................. 190
7.2 Gene expression patterns ................................................................................ 193
7.3 Expression patterns after Wnt deregulation .................................................... 198
7.4 Flow cytometry as a new analytical tool for Hydractinia cell biology ......... 201
7.5 Concluding remarks ....................................................................................... 203

8 References ......................................................................................................... 204

9 Appendix ............................................................................................................ 216
9.1 Solution recipes .............................................................................................. 216
9.2 Gene data ........................................................................................................ 219
9.2.1 Phylogenetic analysis .............................................................................. 219
9.2.2 Wnt1 ............................................................................................... 222
9.2.3 Wnt2 ............................................................................................... 225
9.2.4 Wnt5B ............................................................................................. 229
9.2.5 Wnt7 ............................................................................................... 233
9.2.6 Wnt8 ............................................................................................... 237
9.2.7 Wnt9/10 ......................................................................................... 241
9.2.8 Wnt11A ......................................................................................... 245
9.2.9 Wnt11B ......................................................................................... 249
9.2.10 Wnt16 ........................................................................................... 253
9.3 Web sites used ..................................................................................... 256
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Finally, I would like to give a big thanks to my family. Particularly my parents, Lucie and Heinz Jürgen Hensel, who always supported me, stood behind me and were patient with me when I talked a lot of scientific “nonsense”. I would never have been able to go on and follow my dreams without knowing that you will always be there for me.
Summary of Content
Canonical Wnt signalling controls many developmental processes in metazoans including primary axis formation in embryonic development and stem cell decision-making. The evolutionary history of Wnt signalling and its ancestral role have been of great interest but are not well understood.

During my study I examined Wnt signalling in Hydractinia echinata, a member of the basal metazoan phylum Cnidaria. To date, only two Wnt ligands had been described in this animal, Wnt3 and Wnt5A. However, it was expected that additional Wnt genes should be encoded by this animal’s genome, as past studies carried out in other cnidarians revealed genes belonging to nearly all known Wnt subfamilies.

I identified 9 additional Wnt genes in Hydractinia and analysed their coding sequences and their expression pattern throughout the entire life cycle of this animal.

Phylogenetic analysis of all Hydractinia predicted Wnt proteins, together with other published and unpublished sequences from cnidarians and bilaterians, was performed by Dr. Paulyn Cartwright. The resulting phylogenetic trees contradict the widely accepted hypothesis of pre-bilaterian Wnt subfamily divergence. Our data is consistent with lineage-specific ramification of a small number of ancestral Wnt homologues.

In situ hybridisation of the novel Hydractinia genes revealed non-conserved expression pattern of Wnt genes even within the Hydrozoa. I identified three genes as likely candidates to be associated with Hydractinia stem cell functioning. The genes, Wnt2, -11B and -16 were the only ones that were expressed in the correct anatomical region, the stolons. Furthermore, these genes were also the only ones whose expression was altered following ectopic activation of canonical Wnt signalling, suggesting that they might be functioning either in positive or negative feedback loops.

Finally, I have established new protocols for analysing and sorting Hydractinia cell suspensions by flow cytometry and fluorescence activated cell sorting (FACS). These protocols open up new directions of research and enable qualitative and quantitative analyses of specific genetic manipulations in Hydractinia.
Declaration of contribution

I declare that this thesis is all my work and that I did not obtain a degree at the National University of Ireland Galway or elsewhere on the basis of this work.

My project was funded by Beaufort Biodiscovery Grant and Science Foundation Ireland (SFI).

This Beaufort Marine Research Award is carried out under the Sea Change Strategy and the Strategy for Science Technology and Innovation (2006-2013), with the support of the Marine Institute, funded under the Marine Research Sub-Programme of the National Development Plan 2007–2013.

All work presented in this thesis was performed by me with the following exceptions:

Chapter 1:
Figure 1.3.4 was reproduced with permission from the publication Millane R.C., Kanska J., Duffy D.J., Seoighe C., Cunningham S., Plickert G. & Frank U. (2011) “Induced stem cell neoplasia in a cnidarian by ectopic expression of a POU domain transcription factor.” Development 138, 2429-39.
Figure 1.3.6: The schematic was adapted with permission from the publication Technau U. & Steele R.E. (2011) “Evolutionary crossroads in developmental biology: Cnidaria.” Development 138, 1447-58.

Chapter 3:
Figures 3.12.1 and 3.12.2: Phylogenetic analysis of the identified Hydractinia Wnt genes was performed by Dr. Paulyn Cartwright (University of Kansas).

Chapter 6:
Figure 6.1.1 was reprinted from the Journal of Immunological Methods, Vol /edition number 288 Jahan-Tigh R.R., Ryan C., Obermoser G. & Schwarzenberger K, “Flow Cytometry.”, Pages No. 111-121, Copyright (2012), with permission from Jahan-Tigh et al. 2012.
Figure 6.1.2 was reprinted from the Journal of Immunological Methods, Vol /edition number 288, van Rijt L.S., Kuipers H., Vos N., Hijdra D., Hoogsteden H.C. & Lambrecht B.N, “A rapid flow cytometric method for determining the cellular
composition of bronchoalveolar lavage fluid cells in mouse models of asthma.", Pages No. 111-121, Copyright (2004), with permission from Elsevier.

All FACS experiments were carried out by Dr. Shirley Hanley (staff of REMEDI) with from me provided cell suspensions; this includes Figure 6.3.1 and Figure 6.3.6.

The picture in Figure 6.3.4 was taken by Dr. Cathriona Millane.
### List of Abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>AER</td>
<td>apical ectodermal ridge</td>
</tr>
<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatosus polyposis coli</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl-phosphate</td>
</tr>
<tr>
<td>cat. no.</td>
<td>catalogue number</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>cRNA</td>
<td>complementary RNA</td>
</tr>
<tr>
<td>CDS</td>
<td>Coding DNA Sequence</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>double distilled water</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>Dsh</td>
<td>dishevelled</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial to mesenchymal transformations</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GSK3 β</td>
<td>glycogen synthase kinase 3β</td>
</tr>
<tr>
<td>H₂O</td>
<td>water</td>
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<td>hyb.</td>
<td>hybridisation</td>
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<td>i-cell</td>
<td>interstitial cell</td>
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<td>ICH</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IPG</td>
<td>Isopropylthio β-D-galactoside</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terinate Kinase</td>
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<tr>
<td>LRP</td>
<td>lipoprotein related protein</td>
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<td>MET</td>
<td>mesenchymal to epithelial transformations</td>
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<td>messenger RNA</td>
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<tr>
<td>NCBES</td>
<td>National Centre for Biomedical Engineering Science</td>
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<tr>
<td>NTP</td>
<td>nucleoside triphosphate</td>
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<td>PCP pathway</td>
<td>planar cell polarity pathway</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>Pln</td>
<td>Polynem</td>
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<td>PFA</td>
<td>paraformaldehyde</td>
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<tr>
<td>RACE</td>
<td>Rapid Amplification of cDNA Ends</td>
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<tr>
<td>RAXML</td>
<td>Randomized Axelerated Maximum Likelihood</td>
</tr>
<tr>
<td>Acronym</td>
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<td>RNA</td>
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<td>Universal Reverse Primer</td>
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List of Figures

Chapter 1
Figure 1.1.1: Simplified illustration of canonical Wnt signalling.
Figure 1.1.2: Schematic comparison of canonical and PCP Wnt-pathways.
Figure 1.1.3: Schematic of Ca^2+ - dependent Wnt pathway.
Figure 1.3.1: A colony of *Hydractinia echinata* encrusting a gastropod shell which is usually inhabited by hermit crab.
Figure 1.3.4: Life cycle of *Hydractinia echinata*.
Figure 1.3.3: One day old primary polyp (top view)
Figure 1.3.4: May-Grünwald staining of stolons of *Hydractinia echinata*
Figure 1.3.5: Male adult colony of *Hydractinia echinata*
Figure 1.3.6: Schematic illustration of Hydrozoa body wall.

Chapter 3
Figure 3.3.1: Alignment of *Hydractinia echinata* Wnt1 protein and Wnt1 proteins from different organisms.
Figure 3.3.2: Alignment of *Hydractinia echinata* Wnt1 protein and different *Hydra* Wnt proteins.
Figure 3.3.3: Magnification of the Wnt1 cluster determined in phylogenetic analysis of hydrozoan Wnt proteins
Figure 3.4.1: Alignment of *Hydractinia echinata* Wnt2 protein and Wnt2 proteins from different organisms.
Figure 3.4.2: Alignment of *Hydractinia echinata* Wnt2 protein and different *Hydra* Wnt proteins.
Figure 3.4.3: Magnification of the Wnt2 cluster determined in phylogenetic analysis of hydrozoan Wnt proteins.
Figure 3.5.1: Alignment of *Hydractinia echinata* Wnt5B protein and Wnt5 proteins from different organisms.
Figure 3.5.2: Alignment of *Hydractinia echinata* Wnt5B protein and different *Hydra* Wnt proteins.
Figure 3.5.3: Magnification of the Wnt5B cluster determined in phylogenetic analysis of hydrozoan Wnt proteins.
Figure 3.6.1: Alignment of *Hydractinia echinata* Wnt7 protein and Wnt7 proteins from different organisms.
Figure 3.6.2: Alignment of *Hydractinia echinata* Wnt7 protein and different *Hydra* Wnt proteins.
Figure 3.6.3: Magnification of the Wnt7 cluster determined in phylogenetic analysis of hydrozoan Wnt proteins.
Figure 3.7.1: Alignment of *Hydractinia echinata* Wnt8 protein and Wnt8 proteins from different organisms.
Figure 3.7.2: Alignment of *Hydractinia echinata* Wnt8 protein and different *Hydra* Wnt proteins.
Figure 3.7.3: Magnification of the Wnt8 cluster determined in phylogenetic analysis of hydrozoan Wnt proteins.

Figure 3.8.1: Alignment of *Hydractinia echinata* Wnt9/10 protein and Wnt9 and Wnt10 proteins from different organisms.

Figure 3.8.2: Alignment of *Hydractinia echinata* Wnt9/10 protein and different *Hydra* Wnt proteins.

Figure 3.8.3: Magnification of the Wnt9/10 cluster determined in phylogenetic analysis of hydrozoan Wnt proteins.

Figure 3.9.1: Alignment of *Hydractinia echinata* Wnt11A protein and Wnt11 proteins from different organisms.

Figure 3.9.2: Alignment of *Hydractinia echinata* Wnt11A protein and different *Hydra* Wnt proteins.

Figure 3.9.3: Magnification of the Wnt11A cluster determined in phylogenetic analysis of hydrozoan Wnt proteins.

Figure 3.10.1: Alignment of *Hydractinia echinata* Wnt11B protein and Wnt11 proteins from different organisms.

Figure 3.10.2: Alignment of *Hydractinia echinata* Wnt11B protein and different *Hydra* Wnt proteins.

Figure 3.10.3: Magnification of the Wnt11B cluster determined in phylogenetic analysis of hydrozoan Wnt proteins.

Figure 3.11.1: Alignment of *Hydractinia echinata* Wnt16 protein and Wnt16 proteins from different organisms.

Figure 3.11.2: Alignment of *Hydractinia echinata* Wnt16 protein and different *Hydra* Wnt proteins.

Figure 3.11.3: Magnification of the Wnt16 cluster determined in phylogenetic analysis of hydrozoan Wnt proteins.

Chapter 4

Figure 4.1.1: Schematic illustration of transcription and translation during protein synthesis.

Figure 4.3.1: *Wnt1* expression detected by *in situ* hybridisation during embryonic development.

Figure 4.3.2: Wnt2 expression during embryonic development observed after *in situ* hybridisation.

Figure 4.3.3: Expression of *Wnt5B* during embryonic development.

Figure 4.3.4: The *Wnt7* homologue could be detected uniformly expressed in embryonic development.

Figure 4.3.5: Expression pattern of *Wnt8* observed after *in situ* hybridisation.

Figure 4.3.6: Expression pattern of Wnt9/10 during embryonic development.

Figure 4.3.7: *Wnt11B* expression during embryonic development.
Figure 4.3.8: Expression of Wnt16 during embryonic development.
Figure 4.4.1: Expression of Wnt1 detected by in situ hybridisation at the larva stage.
Figure 4.4.2: Expression of Wnt5B at the larva stage.
Figure 4.4.3: Detection of Hydractinia echinata Wnt7 ligand by in situ hybridisation at larva stage.
Figure 4.4.4: Expression of Wnt9/10 at larva stage.
Figure 4.4.5: Expression pattern of Wnt16 ligand observed after in situ hybridisation.
Figure 4.5.1: Wnt1 expression detected by in situ hybridisation of metamorphosis induced larva.
Figure 4.5.2: Wnt2 expression observed in induced larva stage after in situ hybridisation.
Figure 4.5.3: Wnt5A expression detected by in situ hybridisation of metamorphosis induced larva.
Figure 4.5.4: Expression pattern found after in situ hybridisation with the Wnt7 homologue of Hydractinia echinata.
Figure 4.6.1: Wnt2 in situ hybridisation performed on young colonies.
Figure 4.6.2: Expression pattern observed after in situ hybridisation targeting the Hydractinia Wnt11A ligand.
Figure 4.6.3: Wnt16 expression pattern observed in young colonies of Hydractinia echinata.
Figure 4.7.1: Wnt1 expression in feeding polyps.
Figure 4.7.2: Wnt2 expression in feeding after in situ hybridisation.
Figure 4.7.3: Wnt7 expression pattern observed after in situ hybridisation of adult feeding polyps.
Figure 4.7.4: Expression pattern observed in feeding polyps of the Wnt9/10 ligand.
Figure 4.7.5: Expression pattern observed after in situ hybridisation targeting the Hydractinia Wnt11B ligand.
Figure 4.7.6: Wnt16 expression detected by in situ hybridisation in adult feeding polyps.
Figure 4.8.1: Wnt2 expression pattern in male sexual polyps after in situ hybridisation.
Figure 4.8.2: Wnt16 expression pattern observed in male sexual polyps after in situ hybridisation.
Figure 4.9.1: Schematic drawing of Wnt gene expression throughout the life cycle.

Chapter 5
Figure 5.2.1.1: EdU staining after azakenpaullone treatment
Figure 5.2.2.1: In situ hybridisation targeting Wnt1 after azakenpaullone treatment.
Figure 5.2.2.2: Expression of Wnt2 detected by in situ hybridisation after azakenpaullone treatment.
Figure 5.2.2.3: Wnt5B expression detected by in situ hybridisation after azakenpaullone treatment.
Figure 5.2.2.4: Wnt7 expression after global Wnt activation via azakenpaullone treatment.
Figure 5.2.2.5: *Wnt8* expression after global Wnt activation via azakenpaullone treatment.

Figure 5.2.2.6: *In situ* hybridisation targeting *Wnt9/10* after azakenpaullone treatment.

Figure 5.2.2.7: *In situ* hybridisation after azakenpaullone treatments targeting *Wnt11A*.

Figure 5.2.2.8: *In situ* hybridisation targeting *Wnt11B* after azakenpaullone treatment.

Figure 5.2.2.9: *Wnt16* expression after azakenpaullone treatment detected by *in situ* hybridisation in *Hydractinia echinata*.

**Chapter 6**

Figure 6.3.3: Flow cytometry of utilised size beads displayed as dot plot and histogram.

Figure 6.3.4: Flow cytometry of two independent *Hydractinia echinata* cell suspensions from young colonies, plotted using the parameters of FSC (corresponding to size) and SSC (corresponding to complexity).

Figure 6.3.5: Corresponding hierarchy diagrams of the two flow cytometry data sets shown in Figure 6.3.4.

Figure 6.3.6: Flow cytometry of two independent *Hydractinia echinata* cell suspensions from cut polyps plotted using the parameters of size (FSC) and complexity (SSC).

Figure 6.3.7: Corresponding hierarchy diagrams of the two flow cytometry data sets shown in Figure 6.3.6.

Figure 6.4.1: Contour dot plot and chosen gates for FACS of young colonie cell suspension.

Figure 6.4.2: Light microscopy of sorted cell fractions.

Figure 6.4.3: Light microscopy of sorted “medium” cell fraction

Figure 6.4.4: *Pln* transgenic feeding polyp under blue light excitation.

Figure 6.4.5: Flow cytometry of transgenic *Pln* feeding polyps. (A) Contour dot plot using the parameters of size (FSC) and complexity (SSC).

Figure 6.4.6: FACS of *Pln* transgenic animal cell suspension.

Figure 6.4.7: Light and fluorescent microscopy of GFP** cells of *Hydractinia Pln* transgenic animals, sorted by FACS.

Figure 6.5.1: Flow cytometry of utilised size beads displayed as dot plot and histogram.

Figure 6.5.2: Contour dot plot of *Hydractinia* cell suspension, seawater control.

Figure 6.5.3: Contour dot plots of *Hydractinia* cell suspensions, DMSO controls (upper row) or azakenpaullone treatments (lower row).

**Chapter 9**

Figure 9.2.1.1: Phylogenetic tree of Hydrozoan Wnt genes.

Figure 9.2.1.2: Phylogenetic tree of Wnt genes for different metazoan representatives.

Figure 9.2.2.1: Sequencing results of *Hydractinia Wnt1*.

Figure 9.2.2.2: Full coding sequence of *Hydractinia Wnt1* ligand.

Figure 9.2.2.3: Amino acid sequence of *Wnt1*. 

17
Figure 9.2.2.4: Alignment of *Hydractinia echinata* Wnt1 protein and *Hydra vulgaris* Wnt1 protein.

Figure 9.2.2.5: Alignment of *Hydractinia echinata* Wnt1 protein and different *Hydra* Wnt proteins.

Figure 9.2.3.1: Sequencing results of *Hydractinia* Wnt2.

Figure 9.2.3.2: Coding sequence of *Hydractinia* Wnt2.

Figure 9.2.3.3: Amino acid sequence for Wnt2 gene.

Figure 9.2.3.4: Alignment of *Hydractinia echinata* Wnt2 protein and *Clytia hemisphaerica* WntX1A protein.

Figure 9.2.3.5: Alignment of *Hydractinia echinata* Wnt1 protein and different *Hydra* Wnt proteins.

Figure 9.2.4.1: Sequencing results of *Hydractinia* Wnt5B.

Figure 9.2.4.2: Left agarose gel picture of PCR product. Right lane full coding sequence of *Hydractinia* Wnt5B.

Figure 9.2.4.3: Amino acid sequence of *Hydractinia* Wnt5B.

Figure 9.2.4.4: Alignment of *Hydractinia* Wnt5B protein and *Xenopus laevis* Wnt5B protein.

Figure 9.2.4.5: Alignment of *Hydractinia echinata* Wnt5B protein and different *Hydra* Wnt proteins.

Figure 9.2.4.6: Alignment of published *Hydractinia echinata* Wnt5A protein and newly identified *Hydractinia* Wnt5B protein.

Figure 9.2.5.1: Sequencing results of *Hydractinia* Wnt7 fragment.

Figure 9.2.5.2: Left lane agarose gel picture of PCR product, right lane displays the coding sequence of *Hydractinia* Wnt7.

Figure 9.2.5.3: Amino acid sequence for Wnt7.

Figure 9.2.5.4: Alignment of *Hydractinia echinata* Wnt7 protein and *Hydra vulgaris* Wnt7 protein.

Figure 9.2.5.5: Alignment of *Hydractinia echinata* Wnt5B protein and different *Hydra* Wnt proteins.

Figure 9.2.6.1: Sequencing results of *Hydractinia* Wnt8

Figure 9.2.6.2: Left lane agarose gel picture of PCR product. Right lane coding sequence of *Hydractinia* Wnt8.

Figure 9.2.6.3: Amino acid sequence for Wnt8 gene.

Figure 9.2.6.4: Alignment of *Hydractinia echinata* Wnt8 protein and *Hydra vulgaris* Wnt8 protein.

Figure 9.2.6.5: Alignment of *Hydractinia echinata* Wnt8 protein and different *Hydra* Wnt proteins.

Figure 9.2.7.1: Sequencing results of *Hydractinia* Wnt9/10 fragment.

Figure 9.2.7.2: Left lane agarose gel picture of PCR product. Right lane coding sequence of *Hydractinia Wnt9/10* ligand.

Figure 9.2.7.3: Amino acid sequence for Wnt9/10 gene.
Figure 9.2.7.4: Alignment of *Hydractinia echinata* Wnt9/10 protein and *Hydra vulgaris* Wnt9/10b protein.

Figure 9.2.7.5: Alignment of *Hydractinia echinata* Wnt9/10 protein and different *Hydra* Wnt proteins.

Figure 9.2.8.1: Sequencing results of *Hydractinia* Wnt11A.

Figure 9.2.8.2: Confirmed full coding sequence of *Hydractinia* Wnt11A ligand.

Figure 9.2.8.3: Amino acid sequence for Wnt11A gene.

Figure 9.2.8.4: Alignment of *Hydractinia echinata* Wnt11A protein and *Hydra vulgaris* Wnt11 protein.

Figure 9.2.8.5: Alignment of *Hydractinia echinata* 11A protein and different *Hydra* Wnt proteins.

Figure 9.2.8.6: Alignment of *Hydractinia echinata* Wnt11A and Wnt11B protein.

Figure 9.2.9.1: Sequencing results of *Hydractinia* Wnt11B fragments.

Figure 9.2.9.2: Coding sequence of *Hydractinia* Wnt11B fragment.

Figure 9.2.9.3: Full amino acid sequence for *Hydractinia* Wnt11B protein.

Figure 9.2.9.4: Alignment of *Hydractinia echinata* Wnt11B protein and *Hydra vulgaris* Wnt11 protein.

Figure 9.2.9.5: Alignment of *Hydractinia echinata* 11B protein and different *Hydra* Wnt proteins.

Figure 9.2.10.1: Sequencing results of *Hydractinia* Wnt16.

Figure 9.2.10.2: Full coding sequence of *Hydractinia* Wnt16 ligand.

Figure 9.2.10.3: Amino acid sequence for Wnt16 gene.

Figure 9.2.10.4: Alignment of *Hydractinia echinata* Wnt16 protein, *Hydra vulgaris* Wnt16 protein

Figure 9.2.10.5: Alignment of *Hydractinia echinata* 16 protein and different *Hydra* Wnt proteins.
List of Tables

Table 1.1.1: Wnt ligands so far published for human, Drosophila melanogaster, Platynereis dumerilii, Nematostella vectensis, Hydra spp. and Hydractinia echinata.

Table 2.5.1: Primer sequences. All primers were solved as stock solutions of 100 pmol/µl.

Table 4.9.1: Summary of expression for all identified Wnt ligands in Hydractinia echinata. Wnt ligands published previous to this study are marked with a star. Shown are expression patterns for embryos, larva, metamorphosis induced larva (ind. larva), stolons, feeding polyps and sexual polyps.

Table 5.2.1.2: Statistical comparison of Wnt activated animals with control animals regarding numbers of proliferating cells (EdU*) in stolons after azakenpaullone treatments 1.

Table 6.5.1: Table of percentage rates for gated cell fractions P3 and P4 in relation to all events measured within gate P2. Experiment consists of two independent experiments and 12 replicates each.

Table 7.1.1: Wnt ligands identified for human, Drosophila melanogaster, Platynereis dumerilii, Nematostella vectensis, Hydra spp. and Hydractinia echinata.
1 Chapter One – General Introduction

Discovering the fundamental units that make up multi-cellular organisms led to many questions, which are still being answered to this day. How do cells work together to build a multi-cellular body and how do they differentiate into several different cell types that have distinct functions within an organism (Tsanev & Sendov 1971; Kaiser 2001; Mian & Rose 2011)? How does one single cell form a complex organism such as a human being, and how do they form and maintain the correct numbers of different cell types at the right place at the right time? The role of genes in regulation and communication between cells has been the focus of studies for many years (Birchler & Veitia 2010; Flintoft 2013; Symmons & Spitz 2013; Thomas & Lieberman 2013). Due to the sheer number of complex regulatory networks involved, many biological processes are still poorly understood.

Signalling through several pathways has been suggested to play a crucial role in these processes, including Wnt signalling. Research regarding the Wnt pathway has been conducted for many a year and in a number of different animal models (the Wnt homepage: http://www.stanford.edu/group/nusselab/cgi-bin/wnt/conservation). It has been shown that proper expression of Wnt ligands is essential for development and adult tissue homeostasis (Reya & Clevers 2005). Wnt signalling is well conserved throughout the animal kingdom. Therefore studying this pathway in simple animals is not only easier to conduct, but could also shed a light on the origins of this important signalling pathway.

Additionally, investigating gene relationship has become a more and more important aspect of biology. Throughout the evolution of organisms the number of genes has increased substantially. Genes are often consolidated in gene families (Thornton & DeSalle 2000; Zhang 2003). These gene families consist of several similar genes that share important characteristics and possess similar sequence. The formation of genes with similar sequence is thought to have happened through so called gene duplication of a single original gene (Ohno et al. 1968; Zhang 2003; Taylor & Raes 2004; Conant & Wolfe 2008). This process can occur for example through ectopic recombination, replication slippage (Viguera et al. 2001; Kim & Mirkin 2013) or retro-transposition.
In comparison of related genes it has to be distinguished between so-called homologues, orthologues and paralogues (Fitch 1970; Thornton & DeSalle 2000; Jensen 2001; Gabaldón 2013). Homologous genes are assumed to have derived by decent from a common ancestral gene (Koonin 2005). Genes from different species are classified as orthologous genes when they are thought to have evolved from one gene in their common ancestor, while paralogous genes are related via duplication. However, the definition of paralogues is very general and does not require the genes to reside in the same genome, nor does it give an indication when the duplication event occurred (Koonin 2005).

Resolving relationships between genes might give clues regarding the evolution of organisms and have functional implications.

Therefore, it is befitting to investigate the Wnt pathway in basal animals such as cnidarians. So far only few representatives of the phylum Cnidaria have been examined regarding their Wnt signalling. Surprisingly, members of most Wnt subfamilies have been identified, suggesting that the evolution of Wnt subfamilies predates the splitting of Bilateria and Cnidaria. In *Nematostella vectensis*, a member of the phylum Cnidaria (Anthozoa), genes have been identified for nearly all Wnt subfamilies described in metazoans (Kusserow *et al.* 2005). The only exception is the Wnt9 subfamily, for which no member was observed. Interestingly, no expression of Wnt genes was observed prior to gastrulation (Kusserow *et al.* 2005). In the developing larva, expression of the different Wnt genes was reported to resemble a staggered expression cluster, similar to the Hox cluster observed in bilaterians. It was suggested that the Wnt genes might have a function in establishment of the oral-aboral body axis (Kusserow *et al.* 2005; Lee *et al.* 2006; Petersen & Reddien 2009; Marlow *et al.* 2013). In adult polyps, the majority of identified Wnt genes are described to be expressed in the oral tip of the head (*WntA*, -1, -3, -5, -7, -11 and -16). They are likely to have a role in axis patterning. A function of Wnt genes in stem cell control has not been shown in *Nematostella* so far, as no pluripotent stem cells have been identified.

### 1.1 Wnt signalling

The first two genes described for the Wnt gene family are *wingless* (wg) in *Drosophila melanogaster* (Sharma & Chopra 1976) and *Int-1* (mouse mammary tumour virus integration site 1) in *Mus musculus* (Nusse & Varmus 1982), which were later
combined into the gene family name Wnt (Wingless-related integration site = Wnt). The Wnt pathway is an important signalling mechanism throughout the animal kingdom. It is a gene regulation system linked to many developmental key events and adult processes, such as stem cell fate determination, stem cell proliferation and self-renewal, axis formation, coordinated cell migration, axon guidance and tissue polarity (Clark et al. 2012). Wnt genes code for secreted glycoproteins. Wnt ligand sequence can vary considerably between different paralogues and orthologues, but they all share 22 characteristic cysteine residues, which are responsible for proper protein folding and secretion. They usually consist of 350 to 400 amino acids (Cadigan & Nusse 1997; Clevers 2006). The protein will be palmitoylated before secretion, usually by porcupine. They diffuse and act as morphogenes. Wnt signalling is generally classified into three major subtypes. The most studied is the canonical Wnt pathway, which acts through β-catenin and TCF. Two non-canonical Wnt pathways are described in the literature, the planar cell polarity (PCP) pathway and the Wnt/Ca\(^{2+}\) pathway.

The main effort of studies has been focused on the canonical Wnt pathway (Huelsken & Behrens 2002). It acts through β-catenin destruction when no Wnt stimulus is present (Reya & Clevers 2005). For this a complex consisting of Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3β (GSK3β) phosphorylates cytosolic β-catenin to mark it for degradation by the proteasomes (Barker 2008). While β-catenin is being degraded it does not translocate in the nucleus and therefore downstream target genes are not activated. In cells capable to react to canonical Wnt signalling a receptor belonging to the frizzled family, as well as low density lipoprotein related protein (LRP) co-receptor are present in the cell membrane (Mao et al. 2001). It has been shown that frizzled receptors comprise a cysteine rich extracellular domain that is sufficient to bind Wnt ligands (Lin et al. 1997). When a Wnt ligand binds to the frizzled receptor, dishevelled protein and LRP become differentially phosphorylated. It is not yet fully understood how the phosphorylation of dishevelled (Dsh) functions during the initiation of the Wnt cascade, but at the same time Axin binds to the LRP protein and forms a membrane complex, thereby inhibiting the “destruction complex” for β-catenin (Figure 1.1.1). The levels of β-catenin can then accumulate within the cytoplasm and translocate to the cell nucleus, where target gene transcription can be regulated with its partners of the T cell-specific transcription factor/lymphoid enhancer-binding factor 1
(TCF/LEF) family. The β-catenin replaces a repressor protein (Groucho) in the protein complexes present in the cell nucleus and transforms the complex to an activator (Daniels & Weis 2005) These repressor complexes are also a possible explanation for the diverse functions regulated by Wnt signalling. As the different cell types might have different participants within the repressor complexes responding to the β-catenin, different responses might be triggered.

![Figure 1.1.1: Simplified illustration of canonical Wnt signalling. In cells without Wnt stimulus, a destruction-complex consisting of Axin, APC (adenomatous polyposis coli) and glycogen synthase kinase 3β (GSK3β) marks β-catenin for degradation. As soon as a Wnt ligand binds the frizzled receptor, Axin is removed from the destruction-complex and binds to dishevelled (Dsh) and Arrow/LRP5/6. Therefore the destruction-complex is no longer functional, allowing β-catenin levels to increase within the cells and translocate into the cell nucleus. In the cell nucleus β-catenin binds to T cell-specific transcription factor/lymphoid enhancer-binding factor 1 (TCF/LEF) and activates gene transcription.](image)

As mentioned before, there are also two non-canonical pathways described, PCP and Wnt/Ca²⁺- pathway (Gao 2012). In the past years increasing attention was paid to non-canonical Wnt signalling. Some of the participants of canonical Wnt signalling were
reported to have a function in these pathways as well. The frizzled receptor family was actually first referred to in connection to the PCP pathway in *Drosophila melanogaster*. Studies had shown that the described *frizzled* gene has a function in organising the bristles and hairs of the adult *Drosophila* cuticle via the PCP pathway (Vinson et al. 1989). *Dishevelled* was also reported to participate in the PCP pathway in addition to its function in canonical Wnt signalling (Penton et al. 2002). It was shown that different domains of dishevelled are utilised for the different pathways. While for the canonical pathway the most important domain is DIX on the dishevelled protein, the PCP pathway requires the so called DEP domain (Axelrod et al. 1998).

Few other important participants employed in non-canonical Wnt signal transduction are described so far (Gao 2012). These include amongst others Strabismus/-Vangl (Stbm) (Song et al. 2010), the LIM domain protein Prickle (Pk), and Flamingo (Fmi). However, how these proteins interact on mechanistic level within the PCP is not yet fully understood. It is thought, that Wnt ligands in PCP pathway not only bind to frizzled receptor, but that other receptors such as Ror are targeted as well. The binding of Wnt to Ror receptor then leads to phosphorylation of Strabismus/ Vangl in a dose dependent manner. Downstream of Stbm Prickle is recruited, which is supposed to bind Dsh. These two proteins are thought to antagonise each other, which results in asymmetrical protein localisation (Gao 2012). Additionally c-Jun N-terminale Kinase (JNK), Daam1, RhoA and Rok are thought to be necessary for correct PCP signalling (Katoh 2005; Gao & Chen 2010), but the underlying molecular mechanism of the segregation to proximal and distal domains, which is crucial for development of polarisation, remains poorly understood so far. A simplified schematic of the PCP pathway is shown in figure 1.1.2.
Figure 1.1.2: Schematic of canonical and PCP Wnt-pathways. While frizzled receptor and dishevelled act in both, canonical and non-canonical Wnt pathways, signal transduction downstream of these proteins utilise different participants. Canonical Wnt signalling involves Axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β) and β-catenin. Whereas PCP signalling utilised Strabismus/Vangl (Stbm), Prickle (Pk), Ror, c-Jun N-terminale Kinase (JNK), Daam1, RhoA and Rok.

Another described non-canonical Wnt pathway is the Wnt/Ca\(^{2+}\)- pathway (Kühl et al. 2000; Semenov et al. 2007; De 2011). It has yet to be defined whether or not it is an independent pathway, or is actually a part of the PCP pathway. It was suggested that the calcium flux function is a joint part of PCP signalling, as participants of planar cell polarity pathway are also responsible for release of calcium and therefore depict a second cascade. This cascade functions through CAMKII and PKC, but little is known about the underlying mechanism (Komiya & Habas 2008). A simplified schematic of Wnt/Ca\(^{2+}\)-pathway is shown in figure 1.1.3.
Figure 1.1.3: Schematic of Ca^{2+}-dependent Wnt pathway. It is suggested in the literature that the PCP pathway additionally to its direct task, the polarisation of cells through asymmetrical protein localisation, also generates a calcium flux. This calcium flux is then thought to be further utilised by CAMKII and PKC and results in dynamic and complex cellular responses. However, little is known about the mechanisms.

In the past years additional non-canonical pathways have been described (Semenov et al. 2007), complicating the picture of Wnt signalling even further. In a recent study it was even suggested that we have to review the entire knowledge of Wnt signalling, as it is possible that the different Wnt pathways described in the literature are no divergent pathways at all, but act as one signalling network (van Amerongen & Nusse 2009).

But not only do the mechanisms of the different Wnt pathways remain to be fully resolved; it is also still to be determined when the Wnt subfamilies have evolved and how. Members of most Wnt subfamilies have been identified throughout the animal kingdom; examples are given in table 1.1.1. In past studies it was suggested that Wnt subfamilies have evolved very early in the history of animals, predating the split of Cnidaria and Bilateria (Hobmayer et al. 2000; Kusserow et al. 2005; Guder et al. 2006;
Lee et al. 2006). However, these suggestions were based on phylogenetic analysis of very few basal invertebrates, mainly *Hydra* and *Nematostella*.

Another problem is the fact that naming of the so far published Wnt genes is not always stringent. One example of this are the published Wnt genes in *Drosophila melanogaster*. Genes are named as *wingless*, *DWnt2*, *DWnt3/5*, *DWnt4*, *DWnt6*, *DWnt8* and *DWnt10*. According to the Wnt-Homepage (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/drosophila) the genes *DWnt2* and *DWnt4* are actually orthologues for members of the subfamilies Wnt7 and Wnt9, respectively as defined in vertebrates. Furthermore, the gene named *DWnt8/ WntD* has no orthologue in vertebrates, suggesting this gene is rather an orphan Wnt.

Table 1.1.1: Wnt ligands so far published for human, *Drosophila melanogaster*, *Platynereis dumerilii*, *Nematostella vectensis*, *Hydra spp* and *Hydractinia echinata*.

<table>
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<th>Wnt protein</th>
<th>Hydractinia echinata</th>
<th>Hydra spp</th>
<th>Nematostella vectensis</th>
<th>Platynereis dumerilii</th>
<th>Drosophila melanogaster</th>
<th>human</th>
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</thead>
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<td>x</td>
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<td>Wnt10</td>
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<td>Wnt16</td>
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<td>WntA</td>
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To elucidate the relationship of Wnt genes in different species, data from further basal Invertebrates are required.
Chapter One – General Introduction

1.2 Examples of expression patterns and functions of different Wnt ligands

Studying Wnt genes and comparing them to their counterparts in other animal models might lead to a better understanding of this ancient signalling pathway. Wnt has been suggested to play roles in most major processes of development and tissue homeostasis. Therefore it is likely to have a pivotal influence on development of diverse body plans. Additionally many diseases have been linked to improper Wnt signalling. Decrypting how this pathway evolved and how the mechanisms of different Wnt pathway function could result not only in a better understanding how development evolved, but also lead to new approaches how to target diseases.

In this subsection it is endeavoured to highlight the diversity of Wnt expressing tissues and processes in which Wnt signalling has an important role. Although Wnt ligands have been identified throughout the animal kingdom, only a few examples for each Wnt subfamily have been chosen to be presented here. *Drosophila* Wnt genes, with the exception of wingless, are not discussed here, as this organism comprises a loss of many Wnt subfamilies. Whenever referring to human Wnt genes, capital letters have been used for its nomenclature, as throughout the literature human Wnt genes are referred to as WNT. Therefore this study will follow suit using capital letters for human genes. Finally, the expression pattern of the two most commonly studied cnidarian organisms, *Hydra* and *Nematostella*, are also described to complete the compilation.

**Wnt1**

The *Wnt1* gene was first described in mouse as *Int-1* in relation to a discovered mutation, causing swaying of the mouse while movement, which was first described by Lane in 1967 (Thomas et al. 1991). Later studies on the mouse system showed that *Wnt1* expression is crucial for development of the anterior cerebellum, but also other brain parts can be affected when the expression of this gene is improper (Thomas et al. 1991). In 1987 researchers discovered that the *Drosophila melanogaster* homologue of the *Int-1* gene was identical to the previously described *wingless* gene (Rijsewijk et al. 1987). *Wingless* was specified as a segment polarity gene. Loss of function mutations showed phenotypes of the fruit fly missing wings, which lead to the naming of the gene. Also in *Xenopus* the *Wnt1* gen was described to have a function in patterning of the embryo (McMahon & Moon 1989). It is described to act through canonical Wnt
signalling. In cnidarians the \textit{Wnt1} gene expression was detected in the head of polyps (Kusserow \textit{et al.} 2005; Lengfeld \textit{et al.} 2009), suggesting again a function in patterning of the body axis.

\textbf{Wnt2}

In the literature it is suggested that \textit{Wnt2} genes act through canonical Wnt signalling. The human \textit{WNT2} gene was first described in 1988 by Wainwright (Wainwright \textit{et al.} 1988) as an \textit{Int1} related protein. Expression of this gene was observed in several foetal and adult tissues, but did not overlap with the \textit{Int1} expression patterns. Later studies showed expression of this gene in human and mouse tissues of the placenta, in all foetal developmental stages, liver and heart in adult tissues. It was thought to have a role in the development of foetal allantoic communication. Since then multiple studies have suggested that \textit{Wnt2} has a crucial role in organ formation (Poulain & Ober 2011; Miller \textit{et al.} 2012; Onizuka \textit{et al.} 2012), for example by influencing mesenchyme on the ventral side, as well as anterior foregut at embryonic stages in the rat (Miller \textit{et al.} 2012) and is cooperating with \textit{Wnt7b} to define distal progenitors of the lung tissue. In the zebrafish function in liver specification have been shown (Ober \textit{et al.} 2006). The \textit{Xenopus} \textit{Wnt2} homologue was described to be expressed in the anterior neural plate with a function in axis induction. \textit{Wnt2} has also been linked to several diseases such as speech delay inherent to autism and Peyronie's disease (Marui \textit{et al.} 2010; Chien \textit{et al.} 2011; Dolmans \textit{et al.} 2012). In the cnidarian \textit{Nematostella vectensis} \textit{Wnt2} homologue was also identified. Its expression was detected in a large strip in ectodermal cells in the middle of the embryo (Kusserow \textit{et al.} 2005). The Wnt 1/2/4/7 expression cluster in ectodermal cells was hypothesised to have a strong bias towards neuro-ectodermal expression domains, which indicates similarities to the development of the nervous system in vertebrates. In \textit{Hydra} \textit{Wnt2} expression was observed during very early bud formation, indicating functions in early axis formation of the developing bud (Lengfeld \textit{et al.} 2009).

\textbf{Wnt3}

\textit{Wnt3} expression is linked in most animal species with the formation of the primary body axis. Transcripts of the gene were described in discrete dorsal and lateral regions of the diencephalon and the dorsal spinal cord in mouse embryos, as well as low expression levels in the adult brain (Parr \textit{et al.} 1993; Roelink \textit{et al.} 1993). It was also
shown that $Wnt3^-$ mutant mouse embryos failed to form the primitive streak and mesoderm (Liu et al. 1999). Instead undifferentiated ectoderm was extended, before the embryos died during embryogenesis. The human $WNT3$ gene was cloned in 1993 (Roelink et al. 1993). It was shown that loss of $WNT3$ expression for example leads to Tetra-amelia syndrome, which results in limbless development and other abnormalities (Niemann et al. 2004). In *Danio rerio* $Wnt3$ expression was described in developing tailbud, dorsal diencephalon, dorsal midbrain, rhombic lips and the dorsal portions of the spinal cord (Krauss et al. 1992), but not in mesoderm specification as in mouse (Clements et al. 2009). $Wnt3a$ can also take over the function of $Wnt1$ and $Wnt10b$ in midbrain-hindbrain-boundary constriction (Buckles et al. 2004). In the chicken $Wnt3a$ expression was shown to have an important role in apical ectodermal ridge (AER) formation, which is essential for limb bud development (Kengaku et al. 1998). In *Drosophila* no orthologue of vertebrate $Wnt3$ was identified. In *Hydra* $Wnt3$ was shown to have an important role during head formation and axial patterning (Holstein 2013). $Wnt3$ expression in *Nematostella* was observed in cells around the blastopore starting from gastrulation and continues in ectodermal cells of the pharynx (Lee et al. 2006).

**Wnt4**

$Wnt4$ ligands are referred in literature to function through both, canonical and non-canonical Wnt signalling. $Wnt4$ expression was reported in *Mus musculus* during embryonic development dorsally in the spinal cord and in the floor plate (Parr et al. 1993). Another study revealed that $Wnt4$ expression is essential for kidney development by inducing mesenchyme to epithelial transition, which is underlying nephron development during kidney tubulogenesis (Stark et al. 1994). In adult mice expression could be detected in brain and liver (Gavin et al. 1990). Human $WNT4$ could be detected in numerous tissues such as foetal brain, foetal kidney and foetal liver, as well as the adrenal gland, placenta, mammary gland, prostate, spinal cord, stomach, thyroid, trachea, skeletal muscle and small intestine (Peltoketo et al. 2004). $Wnt4$ was also identified in *Danio rerio*, where it is first expressed at the end of gastrulation in a transverse stripe in the anterior neuro-ectoderm (Blader et al. 1996). At the 10 somite stage expression is observed in the brain immediately caudal to the eye and in later developmental stages distinct expression patterns are described throughout brain development, as well as in the floor pate and dorsally near the tailbud. It was suggested that $Wnt4$ inhibits cell movements, similar to $Xwnt5a$ (Ungar et al.
1995). An orthologue of Wnt4 was not identified in Drosophila. The presence of a Wnt4 gene in Hydra is not described. However in Nematostella Wnt4 expression was observed in cells surrounding the blastopore during gastrulation and in larva stages (Kusserow et al. 2005).

Wnt5

Two members of the Wnt5 subfamily were first predicted and cloned in mouse in 1990 (Gavin et al. 1990), Wnt5a and Wnt5b. The genes are described predominantly to function through non-canonical Wnt signalling, but can also function through canonical Wnt signalling (Cha et al. 2008). While Wnt5b expression was observed throughout the embryo and foetus at low levels, Wnt5a showed complex spatial and temporal patterns during early development. Expression was observed in trophoderm giant cells and in the posterior ectoderm, as well as in mesoderm of the early gastrulating embryo (Gavin et al. 1990). At later stages (9.5 days) Wnt5a could be detected in the posterior, neuro-ectoderm, mesoderm and gut endoderm. The CNS ventral proportions of the midbrain also displayed Wnt5a expression. In the two predominantly studied cnidarian model organisms, Hydra and Nematostella, one Wnt5 ligand was described in past studies respectively. In Hydra it was suggested that Wnt5 has a role in bud and tentacle evagination. The expression of this gene could be detected in few cells at the distal tip of tentacles in intact polyps (Philipp et al. 2009). In Nematostella expression of Wnt5 was observed in the most oral regions of endodermal cells (Kusserow et al. 2005).

Wnt6

The presence of a Wnt6 subfamily member was reported for several animal species. It is mainly described to have a function through non-canonical Wnt signalling. In the mouse, expression of Wnt6 was reported throughout foetal development (Gavin et al. 1990), in mammary gland, primary and secondary enamel knots in the tooth bud (Uusitalo et al. 1999), as well as restricted expression in the epithelium of the esophagus (Lickert et al. 2001). Weak expression of Wnt6 was observed in adult brain tissue, but strongly expressed in adult testes (Gavin et al. 1990). Wnt6 was also suggested to activate Wnt4 expression during tubulogenesis of the kidney (Itäranta et al. 2002). During chicken development Wnt6 expression was suggested as a neural crest inducer (García-Castro et al. 2002). Functions in muscle differentiation or
proliferation, feather formation and a role during somite compartmentalisation were also suggested (Rodríguez-Niedenführ et al. 2003). In *Xenopus* Wnt6 expression levels are low until late stages of neurulation. Expression was observed in the epidermis overlying several developing organs, including the eye, heart, and pronephros. The epithelial expression close to specific sites of tissue rearchitecturing suggested a role in mesenchymal to epithelial transformations (MET) or epithelial to mesenchymal transformations (EMT) (Lavery et al. 2008). In human WNT6 expression was described in precursor cells of the endoderm and neural precursor cells, as well as in the intestinal crypt region for stem or progenitor cells (Katoh 2008). Also co-expression with WNT10A in placenta and adult spleen was observed (Kirikoshi et al. 2001b). No Wnt6 gene was described in *Hydra*. In *Nematostella* Wnt6 expression was reported for cells below the blastopore and in endodermal derivatives (Kusserow et al. 2005).

**Wnt7**

Wnt ligands belonging to the Wnt7 subfamily are found in most animal model systems. These ligands are described to have a function for both, canonical and non-canonical, pathways. Generally there are two subtypes of Wnt7 ligands characterised, Wnt7a and Wnt7b. In certain species such as the zebrafish, gene duplication events have taken place resulting in multiple numbers of these subtypes (Beretta et al. 2011). Wnt7a ligands have been discussed regarding functions in formation and patterning of the reproductive tract of mammals (Miller & Sassoon 1998), as well as in dorso-ventral axis patterning in the chick (Kengaku et al. 1998). WNT7A mutations have been linked in humans to severe limb syndromes (Woods et al. 2006). WNT7A function has also been linked to synaptogenesis and axonal remodelling (Hall et al. 2000). In cnidarian models such as *Nematostella* and *Hydra* one homologue is described of the Wnt7 subfamily, respectively. Expression of this gene was observed in the hypostome area in *Hydra*, mainly expressed in endodermal cells throughout the head and few ectodermal cells surrounding the mouth opening. In comparison, in *Nematostella* Wnt7 expression could be continuously detected after completion of gastrulation, restricted to the oral side in a well defined small area of ectodermal cells.

**Wnt8**

Wnt8 ligands have been found and studied in most animal groups. It is mainly described in connection with canonical Wnt signalling. In mice expression can be found
throughout embryonic development, but is no longer present in later stages (Bouillet et al. 1996; Jaspard et al. 2000). In *Xenopus* Wnt8 is suggested to have a function in mesodermal differentiation during normal development (Christian & Moon 1993; Cui et al. 1995). It was also suggested, that Wnt8 directly influences the expression of Hox genes that are essential for normal development of the anterior-posterior axis and the central nervous system in the *Xenopus* embryo (In der Rieden et al. 2010). In *Hydra* Wnt8 expression was observed at the base of tentacles and could be also detected in newly forming buds at the whole pallisading area of evagination. Therefore it was suggested that Wnt8 has a function in formation of tentacles and buds together with Wnt5 (Philipp et al. 2009). In *Nematostella* there seems to be no obvious connection between Wnt8 and Wnt5. The expression of this gene was observed in more aboral domains in endoderm and forming mesenteries at late planula and polyp stages (Kusserow et al. 2005).

**Wnt9**

Members of the Wnt9 subfamilies are suggested to act through both canonical and non-canonical Wnt signalling. In most animal systems two subtypes are described, Wnt9a and Wnt9b, which were previously named as Wnt14 and Wnt15. Wnt9 was suggested to have important functions in vertebrate heart development (Brade et al. 2006). In chicken only a Wnt9a homologue was reported. This gene was discussed to have a pivotal function in joint formation of the limbs (Hartmann & Tabin 2001), as well as in development of the heart (Brade et al. 2006). In *Danio rerio* Wnt9 ligands are suggested to have a role in neural, oral-pharyngeal ectoderm and mesoderm (Cox et al. 2010), as well as in craniofacial patterning (Jezewski et al. 2008). No Wnt9 ligand could be identified in *Nematostella*. In *Hydra* three Wnt ligands that could not clearly be classified between the subfamilies 9 and 10 could be found (Wnt9/10a, Wnt9/10b and Wnt9/10c). The expression of two of them could be observed by *in situ* hybridisation in the head of feeding polyps and forming buds (Lengfeld et al. 2009). The expression of Wnt9/10a was restricted to a small area at the very tip of both adult polyp and forming bud, whereas Wnt9/10c could be detected in a bit broader area on the polyp head and bud. The temporal expression of these two closely related genes is also not exactly identical, as Wnt9/10c could be detected a bit earlier after head regeneration than Wnt9/10a.
**Wnt10**

Wnt10 subfamily members have been reported from many animal systems. They are reported to act through canonical Wnt signalling. The subfamilies comprise two members each in mouse and human. Two Wnt10 homologues have been reported as well for zebrafish. Wnt10a was suggested to have functions in early brain patterning (Kelly et al. 1993), while Wnt10b is discussed to have a function together with Wnt1 in the formation of midbrain-hindbrain boundary. In Nematostella a Wnt10 ligand was identified that is expressed in individual cells in the endoderm (Kusserow et al. 2005). For expression of Wnt9/10 in Hydra see section for Wnt9.

**Wnt11**

Members of the Wnt11 subfamily are described to act through both, canonical and non-canonical Wnt signalling. Wnt11 expression can be detected in *Mus musculus* during embryogenesis from six to eight somite stage (Christiansen et al. 1995). It was suggested in that study, that Wnt11 might have a role in specific muscle development. Wnt11 was also detected in the developing murine kidney (Lechner & Dressler 1997) where it is thought to have functions in ureteric bud tip elongation. Wnt11 was also described in gut development (Lickert et al. 2001) where it is hypothesised to have a role in tissue specification. In human, *WNT11* expression was observed in foetal lung, kidney, adult heart, liver, skeletal muscle, and pancreas (Kirikoshi et al. 2001a). While in human and mouse only one member of this Wnt subfamily was identified, in *Xenopus* and *Danio rerio* two members of the subfamily are described. In *Xenopus* Wnt11 ligand was described to have an important function in the formation of the dorsal-ventral axis (Ku & Melton 1993; Tao et al. 2005). Another study showed that Wnt11 has a pivotal role in heart development in *Xenopus* (Pandur et al. 2002). Garriock (Garriock et al. 2005) published a paper on a second Wnt11-related gene (*Wnt11-R*), suggesting that Wnt11-R and not the previously published Wnt11 gene is the orthologue of mammalian and chicken Wnt11, as expression patterns of this gene are similar to their chicken counterparts. Wnt11 expression was observed in both cnidarian representatives mentioned here. In *Hydra* Wnt11 expression was described in the oral tip of the polyps and in newly formed buds and was suggested to have a role in formation and maintenance of the head-organiser (Lengfeld et al. 2009). In Nematostella Wnt11 expression was reported in cells surrounding the pharynx in planula stage and later in tentacle endoderm (Lee et al. 2006).
**Wnt16**

This Wnt subfamily was described in many other species previously. Members are suggested to act through both, canonical and non-canonical Wnt signalling. In human, two isoforms of WNT16 have been classified. Expression pattern for the different isoforms differed significantly. While WNT16A showed only in the pancreas a significant expression level, the WNT16B isoform was more widely expressed, showing highest levels in the kidney, placenta, brain, heart, and spleen (Fear et al. 2000). In *Xenopus*, Wnt16 expression could be detected from embryonic stage 35 onwards in the hypochord and broadly in the developing eye (Garriock et al. 2007). In mouse, it was proposed that the function of Wnt16 gene is linked to cortical bone thickness and bone mineral density (Zheng et al. 2012). In *Danio rerio* a function of Wnt16 was suggested in early establishment of haematopoietic stem cells (Clements et al. 2011) by controlling Notch signalling. *Wnt16* expression in *Hydra* is reported in cells of the apical half of the polyp head (Lengfeld et al. 2009). In *Nematostella* *Wnt16* is expressed in pharyngeal endoderm (Lee et al. 2006).

**WntA**

The WntA subfamily was identified in certain invertebrates (Holstein 2012). It is yet to distinguish whether WntA ligands act through canonical or non-canonical Wnt signalling, but studies conducted so far often suggest links between Wnt5 and WntA, which would suggest a role in non-canonical Wnt signalling. Although not present in *Drosophila*, this Wnt gene subfamily seems to be conserved in insects and clusters with WntA ligands of other clades, as for example polycheate and Cnidaria (Bolognesi et al. 2008). Expression of this gene was described in *Tribolium* during embryonic development first in the posterior during blastoderm stage, and then additional expression was detected in a mandibular stripe, which quickly changes into small domains on either side of the ventral midline. WntA expression was also observed in the head and appendages, as well as a segmental expression along lateral edges and the labrum (Bolognesi et al. 2008). In the sea urchin (*Stamateris* et al. 2010) and in *Nematostella* (*Kusserow* et al. 2005) a WntA gene was identified as well. In planarians *WntA* is a typical marker for posterior brain (Felix & Aboobaker 2010).
1.3 *Hydractinia echinata* as a model system

The animal model used for this study is *Hydractinia echinata* (Figure 1.3.1). It belongs into the phylum Cnidaria, members of which are attractive model organisms for evo-devo due to their phylogenetic position within the Metazoa. The phylum Cnidaria is considered a sister group to Bilateria, with a last common ancestor thought to have lived over 650 million years ago. Cnidaria consists of the subphyla Medusozoa and Anthozoa. The Anthozoa includes corals, sea anemones and sea pens. For research regarding evo-devo, the best studied representatives of the anthozoan models are *Nematostella vectensis* and *Acropora millepora*. The anthozoan life cycle consists of planula and polyp stage. The Medusozoa, in addition to those two life stages, also comprises of a medusa stage. The medusa stage, when present, is the sexually reproducing stage. Included in the subphyla are the classes of Hydrozoa, Scyphozoa and Cubozoa. The most studied representatives of the class Hydrozoa are *Hydractinia* spp., *Clytia hemisphaerica*, *Hydra* spp. and *Podocoryne carnea*. A
representative of the class Scyphozoa used in research is *Aurelia aurita*. A member of box jellyfish, Cubozoa, is *Chironex fleckeri*.

*Hydractinia echinata* is a European North Atlantic hydrozoan. It is an exciting model system to study questions of basic developmental biology, regeneration and cell fate determination. Its simplicity combined with the amenability to perform genetic manipulation make it suitable as an experimental model (Plickert *et al.* 2012). There are about 30 known species of *Hydractinia* worldwide. *Hydractinia echinata* is a marine hydroid-polyp that can be found in the European north-eastern Atlantic Ocean (Fleming 1828) encrusting the shells of hermit crabs. Colonies of *Hydractinia echinata* occur around the coasts of Great Britain and Ireland where these crabs can be found. The lifecycle of *Hydractinia* is shown in Figure 1.3.2. The development from fertilised egg to planula larva takes about three days (Plickert *et al.* 1988). In this stage the animal can crawl to reach new habitats and metamorphose to a primary polyp. In nature the trigger for metamorphosis is given by certain bacteria on the shells of hermit crabs. The signal can be simulated in the laboratory by incubation with Cs\(^{2+}\) ions at a concentration of 116mM for about three and a half hours.
The larva is polarised along an anterior-posterior axis where the direction of movement and the position of most neurons define anterior. It can attach with the anterior side to a substrate and metamorphose within 24 hours into a primary polyp (Figure 1.3.3) with outgrowing stolons (aboral structures).
Hence, the larval anterior pole gives rise to aboral structure whereas the posterior pole develops into the polyp head. After completion of metamorphosis the animal can catch prey with the newly formed tentacles and food is digested in the gastric cavity. The stolons keep elongating and in more or less regular distances new polyp buds will form. The stolonal network connects the entire colony and is also the residence of the majority of stem cells, known as interstitial cells (i-cells), which occur interspersed between epidermal cells and provide progenitors to all cell lineages (Figure 1.3.4) (Müller et al. 2004; Teo et al. 2006). To show migratory properties of i-cells and location in the stolons, treatments of mitomycin C were performed on colonies, to deplete i-cell populations. This was then followed by grafting experiments and Giemsa staining, to show that recovery of the treated colonies was due to emigrated i-cells from the grafted tissue (Müller et al. 2004).
Figure 1.3.4: May-Grünwald staining of stolons of *Hydractinia echinata*, highlighting interstitial cells (Arrows) interspersed within the epidermis. Scale bar: 50 µm.

Food particles and cells can move within the colony through the stolonal system. After about three months the colony becomes sexually mature. A mature colony consists of feeding, sexual and defending polyps, which are all one genetic clone.

The feeding polyps, which are also called gastrozooids (Figure 1.3.5 black star), consist of a smooth and long body column. At their oral side there is a mouth surrounded by tentacles, with which the animal captures prey and inserts it into the gastric cavity. The tentacles are armed with large numbers of stinging cells, the so-called cnidocytes or nematocytes, which are unique to the Cnidaria and lead to the name of this phylum. The stinging cells contain a post-Golgi capsule, called nematocyst or cnidocyst, containing an inverted tubule filled with venom. Nematocysts can discharge upon a mechanical/chemical stimulus and inject venom into their target organism, either for feeding or defensive purpose in order to paralyse it. Sexual polyps, also called gonozooids, bear the gonads, which represent a reduced medusa stage (Figure 1.3.5 blue star). Their gonads are filled with sperm or oocytes. Two types of defensive polyps can be found on mature colonies, dactylozooids and tentaculozooids.
These polyps possess high numbers of stinging cells and exhibit reduced tentacles. Each colony expresses a single sex, thought to be genetically determined.

Like all cnidarians, *Hydractinia* is a diploblastic animal. The body wall consists of epidermal and gastrodermal epithelial layers which are only separated by a thin extracellular matrix called the mesogloea. Both body layers initially arise from embryonic ecto- and endoderm, respectively. Indeed, it has been common practice in cnidarian literature to refer to the adult epidermis and gastrodermis as “ectoderm” and “endoderm”, respectively. At least in *Hydractinia* this is problematic, because i-cells (originally endodermal) migrate to the epidermis where they give rise to many cell types. Hence, the usage of “ectoderm” and “endoderm” should be limited only to the embryonic stages. A schematic illustration of a typical Hydrozoa body wall is shown in Figure 1.3.6 (Technau & Steele 2011). Each layer is formed from one sheet of epitheliomuscular cells. Other cell types, including stem cells, reside in the interstitial spaces. Stem cells in *Hydractinia* are called interstitial cells or i-cells (Millane et al. 2011). They occur throughout the whole animal, but are most concentrated in the stolonal epidermal interstices (Frank et al. 2001; Müller et al. 2004). They are small,
round or spindle shaped and about seven to ten micrometres in diameter (Müller 1968; Plickert et al. 1988). Their nucleus is large and takes up a large proportion of the cell. These i-cells share many features with stem cells of higher organisms in morphology and gene expression. They underlie the regenerative ability and normal growth in the animal and are thought to be pluripotent throughout the entire life of *Hydractinia* (Müller et al. 2004). However, the identification of these cells is mainly based on their morphology and little is known regarding gene expression safe-guarding stem cell properties.

![Figure 1.3.6: Schematic illustration of Hydrozoa body wall. Imbedded within epitheliomuscular cells in the gastroderm is secretory cells (gland cells) can be found. In interstitial spaces within the ectoderm i-cells and nematoblast occur. Nematocytes and sensory neurons penetrate through the ectoderm. Source: (Technau & Steele 2011)](image)

Few different cell types are present in *Hydractinia* (Plickert et al. 1988). These cells are mainly identified by their morphology and positioning within the animal, but also expression of certain genes can be assigned to cell types. For example actin expression can be observed in epithelial cells in *Hydractinia* (Künzel et al. 2010; Millane et al. 2011). Sub-fractions of neurons can be identified by expression of for example RFamide, or LWamides; (Katsukura et al. 2003; Plickert et al. 2003). Pan-neuronal gene expression, however, has not been shown in *Hydractinia* so far.
Gland cells within the gastrodermis secrete enzymes and hormones into the gastric cavity that help for example to digest prey (Plickert et al. 1988). In the epidermis neuroblasts can be found in small cell clusters, which are progenitors belonging to the neural lineage. Few types of neurons and nematocytes develop from these cells (Müller et al. 2004). The nematocytes or cnidocytes, also called stinging cells, are a unique cell type only found in Cnidaria. They are mechano-sensory cells that contain a distinct structure called nematocyst. Chemical or physical stimulus induces the ultrafast ejection of a coiled thread and thereby also transfers venom into a target. High numbers of those cells are present in the tentacles. Due to the possession of stem cells that can produce all cell types throughout the whole lifespan the animal has an extraordinary high regenerative ability. If injured or even cut into smaller pieces the animal can restore its normal shape. Even after full dissociation a fully functional animal can reform.

All these characteristics as well as the fact that embryos are produced on a daily basis under good culture conditions and appropriate light/dark cycle make *Hydractinia* a valuable animal model for research regarding developmental questions and patterning events (Frank et al. 2001). Many studies have been undertaken to investigate important processes such as embryonic development, stem cell fate determination or body patterning, to name just a few (Rebscher et al. 2008; Duffy et al. 2010; Künzel et al. 2010; Millane et al. 2011). Furthermore, many experimental methods have been established for *Hydractinia* to investigate gene expression and functionality, such as *in situ* hybridisation (ISH), fluorescent *in situ* hybridisation (FISH), immunohistochemistry, RNAi and transgenesis.
1.4 Aims of the research

To this day, little is known about the ancient functions of the Wnt pathway and how Wnt signalling evolved to be a critical player in the regulation of so many processes during embryonic development and adult tissue homeostasis. Studying Wnt signalling in a basal animal will directly address this issue and may also identify new roles for Wnt.

Initial Wnt-related studies in the Cnidaria were done on the evolutionarily derived freshwater polyp *Hydra* and on the sea anemone *Nematostella*. Since then, substantial amount of functional work was done in *Hydractinia*, which is probably a better representative of the class Hydrozoa, but so far only two Wnt ligands have been described in this animal. Given that in other cnidarian models nearly all 12 Wnt subfamilies are reported, it was to expect that additional Wnt ligands should be encoded by this animal’s genome. *Nematostella* possesses ligands belonging to all Wnt subfamilies with the exception of Wnt9 (Kusserow et al. 2005; Guder et al. 2006) and in *Hydra* 11 ligands have been identified (Lengfeld et al. 2009).

Therefore, I decided to identify and clone all *Hydractinia* Wnt ligands and to study their expression pattern throughout the life cycle. In a past study it was suggested, that stem cell decision-making is controlled by canonical Wnt signalling in *Hydractinia*. None of the studied Wnt ligands so far displays expression in the stolons, where the majority of the stem cells reside. Therefore I also aimed at identifying possible candidates of stem cell controlling Wnt ligands. Finally I attempted to investigate cellular changes after Wnt deregulation and for that I had to establish new ways to quantify cells in this animal.

The specific aims of my work were as following:

1. Identification, cloning and sequence analysis of all Wnt genes in *Hydractinia echinata*.
2. Investigating the expression patterns of these genes throughout the life cycle.
3. Studying the effect of Wnt deregulation on stolonal stem cells.
4. Establishment of flow cytometry (FC) as an analytical tool for *Hydractinia* cell suspensions. This includes:
   - Optimisation of a dissociation protocol for *Hydractinia* cells.
Optimisation of cytometer parameters for the use of *Hydractinia* cell suspensions and analysis of WT suspensions regarding distinctive cell populations.

Analysing the total cell complement following ectopic Wnt activation.
2 Chapter Two - Material and Methods

2.1 Animal culture

2.1.1 Culturing

The animal model used for this study is *Hydractinia echinata*. It is a marine hydrozoans polyp, building polymorphic colonies on the shells of hermit crabs. *Hydractinia* can be found around the coast of Ireland and Britain. The animals used were sampled in the Galway Bay, Ireland.

The culturing was performed in a temperature stable room, set to 19 °C. Natural seawater was used and a light/dark cycle of 14/10 h was applied.

Animals were fed 5 days a week with *Artemia salina* nauplii for 1 hour in the morning and then transferred in fresh seawater. After five to six hours later the shells were washed under a jet of seawater.

Crabs were fed with a piece of cod while the *Hydractinia* were in the solution of *Artemia salina* nauplii.

Embryos were collected every day about two hours after onset of the light.

2.1.2 Metamorphosis

To metamorphose animals, three to four day old larva were incubated in CsCl/Seawater of a final concentration of 116mM for three and a half hours. Afterwards the induced larva were washed three times with seawater (SW) and then settled on glass cover slips to finish metamorphose into primary polyps for 24 hours.
Chapter Two - Material and Methods

2.2 Protocols

2.2.1 Total RNA extraction
All steps were performed with RNase free plasticware and solutions.

2.2.1.1 Small sample amounts
RNeasy MiniKit from Qiagen (cat. no. 74104) in combination with RNase free DNase set (cat. no. 79254) was used for small sample amounts.

Tissue was handled according to manufacturer protocol, either used fresh or stored in RNAlater at 4°C till extraction.

1) Remove all liquid from the tissue and add 600 μl of RLT buffer containing 10 μl/ml β-Mercaptoethanol.
2) Disrupt tissue by vortexing till lysate is fully homogenised.
3) Centrifuge at full speed for 3 min.
4) Carefully transfer supernatant and add same volume of 70% Ethanol. Mix by pipetting.
5) Transfer up to 700 μl of the sample into the spin column, placed in a 2 ml collection tube and spin for 15 sec at 8000g (10000rpm). Discard flow through and repeat step till the entire RNA sample is bound to spin column.
6) Add 350 μl of buffer RW1 to the column and spin for 15 sec at 8000g. Discard flow through.
7) Add 10 μl of DNase 1 stock solution to 70 μl of buffer RDD, mix gently and transfer all to RNA bound to the spin column. Incubate for 15 min at room temperature.
8) Add 350 μl of buffer RW1 to the column and spin for 15 sec at 8000g. Discard flow through.
9) Add 500 μl of buffer RPE to the column and spin for 15 sec. Discard flow through.
10) Add 500 μl of buffer RPE to the column and spin for 2 min at 8000g.
11) Transfer spin column to new 2ml collection tube. Spin for another 1 min.
12) Transfer spin column in a 1.5ml collection tube. Add 50 μl of RNase free water in the middle of the column to elute the bound RNA. Spin for 1 min at 8000g.
13) If yield is supposed to be high, repeat step 12 with the eluate.

Perform ammonium acetate precipitation if concentration is too low for reverse transcription.
2.2.1.2 Big sample amounts

1) Homogenise the sample in 1 ml of lysis buffer per 25-50 mg tissue. Homogenise by vortexing till complete lysis is achieved.
2) Centrifuge at highest speed for 1 min. Transfer all supernatant, taking care not to transfer any remaining tissue pieces or other bits.
3) Add 100 μl of chloroform per 500 μl supernatant to the sample and vortex for 15 sec, then incubate on ice for 20 min.
4) Centrifuge for 20 min at 4ºC 12000g for phase separation. The upper colourless layer contains the RNA.
5) Transfer the upper layer to a new tube and add same volume of chloroform. Mix well and spin at highest speed for 5 min. Transfer upper layer to new tube and measure the volume.
6) Add 0.5 volume of 1.2 M NaCl/ 0.8 M Na-citrate and 0.6 volume of ice-cold Isopropanol per volume of RNA solution, mix by inverting the tube, then incubate on ice for 30 min to precipitate the RNA.
7) Centrifuge at 12000g for 10 min, discard supernatant. Air-dry the RNA pellet for maximum 5 min.
8) Wash pellet with 400 μl of 4 M LiCl. Centrifuge at 12000g for 10 min to recover pellet.
9) Discard supernatant and wash in 400 μl of 80 % ice-cold ethanol. Centrifuge at 12000g for 2 min.
10) Air-dry the pellet for not longer than 4 min and dissolve in 15-30 μl of DEPC water.
2.2.2 DNase digestion of total RNA

DNase digestion is performed to clean up a RNA sample after extraction without kit. All steps have to be performed under RNase free conditions with RNase free solutions and plasticware. RiboLock can be added to ensure integrity of RNA sample. RQ1 RNase-free DNase from Promega (cat. no. M6101) has been used.

1) Measure volume and concentration of RNA. Per 1 µg RNA 1 µl DNase is required. Set up reaction as following:

\[
\begin{align*}
X \mu l & \text{ RNA sample (1 µg)} \\
1 \mu l & \text{ DNase (1 µl/µg)} \\
2 \mu l & \text{ of 10X DNase buffer} \\
1 \mu l & \text{ RiboLock} \\
Z \mu l & \text{ Water} \\
\end{align*}
\]

\[
\text{= 20 µl; Water and RNA can have a total volume together of maximal 16 µl.}
\]

2) Incubate reaction at 37 °C for 30 min
3) Add same volume (20 µl) phenol (pH 4) and chloroform and mix well.
4) Centrifuge at highest speed for 3 min.
5) Transfer upper aqueous layer to a new tube and add same volume chloroform. Mix well.
6) Spin for 3 min at highest speed.
7) Transfer aqueous layer to a new tube and add 0.1 volume of 3 M sodium acetate pH 4.8, mix well without vortexing. Add 1 volume ice cold Isopropanol, mix well and incubate on ice for 50 min.
8) Spin at highest speed for 10 min.
9) Discard supernatant and wash pellet with 400 µl 75 % ice-cold ethanol for 5 min.
10) Spin sample for 5 min at maximum speed. Discard supernatant and air-dry pellet for maximum 2 min.
11) Dissolve pellet in 20-30 µl RNase free water. Measure the concentration on nanodrop.
2.2.3 Formaldehyde denatured 1.5 % gel for RNA analysis

1) Prepare 10X MOPS electrophoresis buffer:
2) Prepare the gel:
   - Mix 0.75 g agarose and 36 ml H2O (DEPC)
   - Dissolve Agarose by boiling in the microwave until clear
   - Add 5 ml 10X MOPS electrophoresis buffer and 9 ml deionised formaldehyde
   - Pour into gel form with comb and allow gel to set
3) Cover gel in Electrophoresis-chamber with 1X MOPS buffer, using 1ml of 10X buffer to 9 ml DEPC water
4) Prepare RNA sample:
   - Mix RNA at a ratio of 1:1 with 2X RNA loading dye
   - Heat up RNA samples and RNA ladder (RiboRuler™ High Range RNA ladder, Fermentas, cat no. SM1821) for 10 min at 70 ºC
   - Cool down on ice for 2 min
   - Load ladder and RNA samples on RNase free prepared gel. Let run for 30 min at a voltage of 100V
5) Photograph under UV light.

2.2.4 cDNA synthesis

2.2.4.1 RT cDNA was transcribed according to the protocol for Omniscript Reverse transcriptase

Transcriptase (Qiagen cat. no. 205110). Per reaction 1 μg RNA and 1 μl oligo dT primer was used. Mix set up reaction well, and then incubate at 37ºC for 1h 30min.

x μl RNA (1 μg)
2 μl 10X buffer
1 μl oligo dT primer
2 μl 5 mM dNTPs
1 μl RNase inhibitor (diluted to 10u/ μl)
1 μl reverse transcriptase
y μl nuclease free water
----------
20 μl
2.2.4.2 RACE cDNA

RACE cDNA was transcribed according to the protocol for Omniscript Reverse Transcriptase (Qiagen cat. no. 205110). 2 μg RNA per reaction and oligos designed according to SMART™ RACE cDNA amplification kit were used. All utilised gene specific primers had a concentration of 10 pmol/μl.

a) 5’ cDNA synthesis

\[
\begin{align*}
&x \mu l \text{ RNA}^* \\
&1 \mu l \text{ 5’ CDS oligo} \\
&1 \mu l \text{ smart 2} \\
&\text{Top up to a final volume of 10 μl with nuclease free water}
\end{align*}
\]

* amount in μl dependents on RNA concentration

b) 3’ cDNA synthesis

\[
\begin{align*}
&x \mu l \text{ RNA}^* \\
&1 \mu l \text{ 3’ CDS oligo} \\
&\text{Top up to a final volume of 10 μl with nuclease free water}
\end{align*}
\]

1) Incubate for 10 min at 70°C.
2) Cool down on ice for 2 min and spin briefly
3) Add 10 μl of a MasterMix consisting of:

\[
\begin{align*}
&2 \mu l \text{ 10X buffer} \\
&2 \mu l \text{ 5 mM dNTPs} \\
&1 \mu l \text{ RNase inhibitor (diluted to 10u/ μl)} \\
&1 \mu l \text{ reverse transcriptase} \\
&4 \mu l \text{ nuclease free water} \\
&10 \mu l
\end{align*}
\]

4) Mix by pipetting and incubate for 1h 30min at 37°C.
5) Dilute 10X 1st strand product in water.
6) Heat up for 7 min to 72°C. Store cDNA at -20°C.
2.2.5 PCR

2.2.5.1 PCR using GoTaq® DNA polymerase

GoTaq® polymerase (Promega, cat no. M3001) produces sticky end fragments. All utilised gene specific primers had a concentration of 10 pmol/µl.

PCR reaction:
1 µl RT cDNA
2 µl forward primer
2 µl reverse primer
8 µl nuclease free water
7 µl Master Mix
----------------------------
20 µl

Master Mix/reaction:
4 µl go flexi buffer (5X)
2 µl MgCl2
0.4 µl dNTPs
0.4 µl nuclease free water
0.2 µl Taq polymerase (go taq)
----------------------------
7 µl

RACE PCR reaction:
2 µl forward primer
2 µl reverse primer
2 µl cDNA (5’ for 5’ race/3’ for 3’ race)
28.8 µl nuclease free water
15.2 µl Master mix
----------------------------
50 µl

Master Mix/reaction:
10 µl go flexi buffer (5X)
4 µl MgCl2
1 µl dNTPs
0.2 µl Taq polymerase
----------------------------
15.2 µl
For 5' RACE spliced leader primer and a gene specific reverse primer was used. For 3' RACE Universal Primer Mix and gene specific forward primer was used. For amplifying of known sequence gene specific forward and reverse primers were used.

PCR conditions:
1 min 80ºC (Hot start)
3 min 95ºC (enzyme activation)

up to 40 cycles:
30 sec 95ºC
40 sec XºC*  *depends on melting temperature of primers
1 min/kb* 72ºC  *time depends on length of amplifying fragment

2.2.5.2 PCR using Phusion High Fidelity™ DNA Polymerase
Phusion High Fidelity™ DNA Polymerase (Finnzyme, cat no. F530S) produces blunt end fragments. All utilised gene specific primers had a concentration of 10 pmol/µl.

PCR reaction:
1 µl RT cDNA
2 µl forward primer
2 µl reverse primer
9 µl nuclease free water
6 µl Master Mix
----------------
20 µl

Master Mix/reaction:
4 µl go HF buffer (5X)
0.4 µl dNTPs
1.5 µl nuclease free water
0.1 µl Phusion polymerase
---------------------------
6 µl

PCR conditions:
1 min 80ºC (Hot start)
5 min 98ºC (enzyme activation)

up to 40 cycles:
30 sec 98ºC
40 sec XºC*  *depends on melting temperature of primers
1 min/kb* 72ºC  *time depends on length of amplifying fragment
2.2.5.3 Cloning of PCR products with phosphorylated primers

For cloning DNA fragments by ligating with dephosphorylated vector DNA, the fragments should have phosphates on their 5' termini. Since phosphorylation of PCR products by T4 polynucleotide kinase is inefficient, primers should be phosphorylated prior to PCR reaction.

1. Phosphorylation of PCR primers.
Mix the following solutions for each primer required for PCR. The 10x T4 PKase buffer contains 0.7 M Tris-Cl (pH 7.6), 0.1 M MgCl2 and 50 mM dithiothreitol.
0.45 μl primer (100 pmol/μl)
0.45 μl 10x T4 PKase buffer
0.45 μl 10 mM ATP
2.75 μl H2O
0.40 μl T4 polynucleotide kinase
----------------
4.5 μl

Incubate for 1 hour at 37 ºC. Store frozen at -20 ºC.

2. Set up PCR reaction
Mix the following solutions.
5 μl 10x PCR buffer
1 μl template
1 μl phosphorylated primer (forward)
1 μl phosphorylated primer (reverse)
5 μl dNTP mix (2.5mM each)
36.5 μl H2O
0.5 μl thermo stable DNA polymerase
----------------
50 μl

Perform polymerase chain reaction according to required conditions for used polymerase, then purify PCR product by agarose gel electrophoresis or appropriate cartridge.
2.2.6 Sequencing

To verify DNA fragments amplified in PCR reactions, sequencing was performed using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Invitrogen/life technologies™, cat no. 4458688).

1) Two reactions were set up per fragment, each reaction containing either the forward or the reverse primer used in PCR:
   1 μl primer
   2 μl Sequencing Buffer* (5X)
   1 μl Ready Reaction Mix*
   x μl DNA (50-100 ng)
   ----------------
   Top up to 10 μl with nuclease free water

* “Sequencing buffer” and “Ready Reaction Mix” are delivered ready to use with sequencing Kit. Concentration and substances are not defined in the manual.

2) Sequencing PCR was performed under following conditions:
   1 min 98°C
   40 cycles
   10 sec 95°C
   10 sec 50°C
   4 min 60°C

3) Sequencing reaction was stopped as followed:
   Per reaction use:
   2.5 μl H₂O
   1 μl 10 M ammonium acetate
   1 μl 50 mM EDTA (pH 8)
   0.5 μl glycogen
   ------------------
   5 μl

   To 10 μl of sequencing PCR product add 5 μl stopping mix and vortex.

4) Add 45 μl of 100 % ethanol to the sample and mix by vortexing.
5) Incubate samples at room temperature for 1 min and then centrifuge for 5 min at max speed.
6) Remove the supernatant and add 200 μl of 75 % Ethanol, then vortex the sample and centrifuge for 2 min at max speed.
7) Remove the supernatant and air dry the sample.
8) Dissolve the pellet in 20 μl nuclease free water.
The samples were then sent for sequencing analysis to Genomics Core Facility at the Regional Genetics Centre of the Belfast City Hospital.

### 2.2.7 Gel extraction

Gel extraction was performed with Genescript Quick Clean II Gel extraction Kit (Genescript, cat no. L00418) according to manufacturer protocol.

1. After Gel electrophoresis cut the bands of interest with as little Agarose as possible.
2. Weigh the gel slice. Add 3 volumes of binding buffer II to the gel slice (50 mg ≈ 50 μl).
3. Heat at 55 °C for 10 min. Vortex in between to make sure all Agarose is solved.
4. Add 1 volume of Isopropanol to the solution and vortex. Transfer up to 700 μl into the spin column and centrifuge at 6000g for 1 min.
5. Discard flow through and repeat till the entire sample is loaded to the column.
6. Add 500 μl of binding buffer II to the spin column and centrifuge at 12000g for 1 min. Discard flow through.
7. Add 750 μl of washing buffer to the column and spin at 12000g for 1 min. Discard flow through.
8. Spin for 1 min at 12000g to avoid of residual ethanol.
9. Transfer the spin column into a 1.5ml collection tube. Add 30 μl of nuclease free water and incubate for 2 min to elute the DNA. Spin at 12000g for one min.

Measure the concentration. If the concentration is too low, precipitation with sodium-chloride can be performed.
2.2.8 DNA clean up with phenol/ chloroform and precipitation

After gel extraction or enzymatic digestion a cleanup was performed as followed:

1) Add a 1:1 ratio of phenol (pH 7) to sample and mix by vortexing. Add same amount of chloroform and vortex again.
2) Centrifuge at maximum speed for 3 min. Transfer upper aqueous layer into new tube.
3) Add same amount of chloroform as sample to the tube, mix well and then centrifuge again at maximum speed for 2 min.
4) Transfer upper aqueous layer into new tube without touching the lower layers.
5) Add 1/20 volume of 5 M NaCl and 1 μl of glycogen. Mix well without vortexing.
6) Add 2.5 volume of 100 % ethanol and mix well. Precipitate DNA at -20 ºC for about 30 min.
7) Centrifuge the sample for 5 min at maximum speed. Discard the supernatant.
8) Wash DNA pellet with 500 μl of 75 % ethanol. Centrifuge at maximum speed for 2 min.
9) Remove supernatant and air dry pellet for maximum 4 min.
10) Dissolve DNA in 10 - 20 μl nuclease free water.

2.2.9 DNA ligation

For ligation the pGEM-Vector Kit from Promega was used (Promega, cat no. A3600). The reaction for ligation was set up as followed:

\[
\begin{align*}
&x \mu l \text{ PCR product (3:1 concentration excess compared to vector)} \\
&y \mu l \text{ pGEM-t vector (20 – 50 ng)} \\
&5 \mu l \text{ rapid ligation buffer (Promega pGEM-t vector kit)} \\
&1 \mu l \text{ T4 DNA ligase}
\end{align*}
\]

Top up to 10 μl with nuclease free water

Mix by pipetting and leave for 1 hour at room temp.
2.2.10 Cloning
Cloning was performed using either E. coli C600 bacteria (kind gift of Prof. Günter Plickert) or XL-1 blue E. coli

1) Heat up agar plates (already containing Carbenicillin 100 μg/ml) to 37 °C.
2) *** only for blue/white screening*** Spread 40 μl of 40 mg/ml X-gal in dimethylformamide on plate and leave to dry. Then spread 100 μl IPG (stock solution 10 mM) on the plate and leave to dry again before use.
3) Use 10 μl DNA ligation product and add 15 μl of water to bring up to a total volume of 25 μl. Denature for 15 min at 65-70 °C.
4) Incubate on ice for 5 min then centrifuge short.
5) Defrost bacteria on ice. Use 50 μl bacteria to 25 μl diluted ligation mix.
6) Mix gently, and incubate on ice for 10 min.
7) Heat-shock at 42 °C for 90 seconds and put tube back on ice to cool for 5 min.
8) Add 800 μl LB medium without antibiotic and incubate for 45 min.
9) Spread the entire transfected bacteria on warmed plates, dry in 37 °C oven. Then turn the plates around and incubate overnight at 37 °C.

Colonies were analysed by PCR using UFP and URP primer.

2.2.11 Plasmid extraction from bacteria
1) Grow up bacteria in 5 ml LB medium, containing 100 μg/ml of carbenicillin, per colony at 37 °C over night.
2) Centrifuge bacteria for 30 sec at maximum speed. Remove supernatant.
3) Re-suspend bacteria in 100 μl of solution I. Leave for about 5 min at RT
4) Add 200 μl solution II and mix by inverting the tube 10 times. Place tube straight on ice.
5) Add directly 150 μl solution III and vortex.
6) Incubate on ice for 5 min, then centrifuge at maximum speed for 2 min.
7) Place supernatant in new tube and add 400 μl phenol (pH 8) and mix well.
8) Add 400 μl chloroform, mix by vortexing and centrifuge at maximum speed for 3 min.
9) Transfer upper, aqueous layer into a new tube and add 400 μl chloroform. Mix well and centrifuge 3 min at maximum speed.
10) Transfer upper layer into a new tube and mix well with 1 ml 100% ethanol. Centrifuge at maximum speed for 5 min.
11) Remove supernatant and wash with 70 % ethanol. Centrifuge at maximum speed for 3 min.
12) Remove supernatant and air dry pellet.
13) Dissolve in 50 μl nuclease free water.
2.2.12 RNA probe synthesis for *in situ*

All steps were performed using RNase free water and plastics.

As template either linear plasmid containing the gene of interest or PCR product amplified with gene specific primers that have T7 or Sp6 RNA polymerase sequence attached can be used.

If plasmid is used as template, an enzyme digestion has to be performed first. The enzyme chosen has to leave the template fragment either blunt end or with a 5’ overhang.

Important: No 3’ overhangs should be generated by enzymatic digestion or digestion of the enzyme within the gene sequence.

**Bluescript:**
1) For T3 promoter: SacI, SacII, NotI, Xbal, Spel, Bamhl, SmaI, Pstl, EcoRI, EcoRV
2) For T7 promoter: HindIII, Clal, Sall, Xhol, Apal, Kpnl

**pGEM-T:**
1) For Sp6 promoter: Apal, AatII, SphI, Ncol, SacII,
2) For T7 promoter: SpeI, NotI, PstI, Sall, Ndel, SacI, Bstxl, Nsi

For labeling RNA probes DIG labeled NTPs (DIG RNA Labeling Kit, Roche, cat. no. 11175025910) were used.

1) Prepare labelling reaction for each, sense and anti sense, probe separate.

   4 µl 5X transcription buffer (Fermentas)
   0.5 µl RNase inhibitor (Fermentas, RiboLock)
   2 µl 0.2M DTT (Dithiotretiol)
   x µl DNA template (200 ng PCR cleaned band or 1 µg digested plasmid)
   2 µl DIG labelled rNTP mix (Roche)
   1 µl RNA polymerase (e.g. T7/SP6) converts DNA → RNA

   Top up to 20 µl with DEPC treated water

2) Incubate reaction for 2h at 37 ºC
3) Add 1 µl exonuclease and incubate for 30 min at 37 ºC
4) Add 1 µl RNase free DNase and incubate for another 30 min at 37 ºC
5) Clean up RNA probe using ethanol precipitation for RNA
6) Add ammonium acetate to a final conc. of 1M (RNase free) and mix well.
7) Add 2.5 volumes of 100 % ethanol, mix well by inverting the tube. Incubate at room temperature for 45 min.
8) Centrifuge for 5 min at max speed. Remove supernatant.
9) Wash with 70 % ethanol by vortexing.
10) Centrifuge at max speed for 2 min. Remove the supernatant.
11) Air dry pellet for not more than 2 min, then dissolve pellet in 20 μl of RNase free water. Measure concentration on nanodrop.
12) Check RNA probe regarding degradation on 1.5 % denaturing formaldehyde RNA gel using 2 μl sample mixed with 2 μl RNA labelling dye.
13) If integrity of RNA sample is satisfying, mix at a 1:1 ratio with hybridisation mix (see in situ protocol). Store the probe at -70ºC.
2.2.13 Whole mount *in situ* hybridisation

In *in situ* hybridisation was performed according to adapted protocols described by Gajewski and Teo (Gajewski *et al.* 1996; Teo *et al.* 2006). For this around 30 animals per life stage were utilised per experiment. Experiments were replicated twice for each examined gene and life stage independently.

All work had to be performed under RNase free conditions and solutions, to guaranty integrity of RNA probes during hybridisation process.

1) Anesthetise animals with 4 % MgCl$_2$/SW for about 30 min.
2) Fix animals for 1 h at room temperature in 1 ml 4 % PFA-Hepes solution.
3) Wash 3 x 10 min with PBST
4) Bleach animals 10 min each rocking in 25 % /50 % /75 % /100 % / 75 % / 50 % / 25 % Methanol; Dilutions are made with PBST.
   (Animals can be stored frozen in 100 % Methanol at -20 °C)
5) Heat for 15 min at 98°C in PBST to eliminate all natural occurring alkaline phosphatase.
6) Wash with 1X Tetraethylammonium chloride (TEA); 1X TEA+ 6 μl /ml acidic acid; 1X TEA+ 12 μl /ml acidic acid; 1X PBST each for 10 min to reduce background binding of probes.
7) Post-fix with 4% PFA in 1 x PBS at RT for 20 min rocking.
8) Wash animals 3 x 5 min in 1 ml PBST at RT rocking.
9) *Prepare for next day: pre-absorb the antibody in a 1:1000 dilution (Anti-Digoxigenin-AP, cat no. 11093274910, Roche) in 1% BSA/PBST overnight at 4 °C with previously fixed polyps rocking. Antibody solution 1 μl antibody in 1 ml 1% BSA/PBST.*
10) Incubate samples in Blocking Solution for 10 min at RT with rocking. Blocking solution: 2 mg tRNA/ 1 ml PBST.
11) Add equal volume hybridisation (hyb.) mix to the blocking solution and rock for 10 min at RT.
12) Remove solution and add 200 μl of hyb. mix for reaction tubes or 1 ml for small petri-dishes; incubate for 2 h at 50°C in a hybridisation oven, with rocking.
13) Heat hyb. mix containing 4-8 ng of RNA probe per 200 μl for 10 min at 70°C for denaturation, before adding them to the animals.
14) Remove pre-hybridisation mix from samples and replace with 200 μl of hyb. mix containing 4-8 ng of sense or antisense RNA probes (20-40 ng/ml). Hybridise overnight at 45-55°C* in the hyb. oven with rocking.  *Temperature depending on probe.
15) Remove RNA DIG-probes.
16) Wash once in Hyb. Mix at 45-55 °C for 5 min.
17) Wash once with 50% Formamide, 2x SSC, 0.1% Tween for 1 h at 45-55°C.
   For 10 ml:
   5 ml Deionised Formamide
   1 ml 20X SSC
   4 ml DEPC water
   10 μl Tween 20
18) Wash once with 2x SSC, 0.1% Tween for 15 min at 45-55°C.
   For 10 ml:
   1 ml 20X SSC
   9 ml DEPC water
   10 μl Tween 20
19) Wash twice with 0.2x SSC, 0.1% Tween for 15 min at 45-55°C.
   For 10 ml:
   100 μl 20X SSC
   9.9 ml DEPC water
   10 μl Tween 20
20) Wash with 1 x PBST for 5 min at RT with rocking
21) Block with 1% BSA in PBST for a minimum of 1 h at RT with rocking.
22) Centrifuge the pre-absorption mixture and use the supernatant in the chemical
detection reaction. Dilute further from 1:1000 to 1:2000 by adding same amount of
1% BSA/PBST
23) Incubate for 2 h at RT in 1:2000 diluted pre-absorbed anti-DIG-AP conjugated Fab
fragments in PBST/1% BSA.
24) Wash 4 times for 20 min with 1 ml 1X PBST, RT, rocking, or 3 times for 20 min and
the last wash over night rocking at 4°C
25) Wash 3 times for 5 min in freshly made alkaline phosphatase buffer
(AP-buffer), 1 ml per tube/ petri-dish.
26) Stain with BCIP/NBT staining solution (recipe see in Appendix) in the dark, 1 ml per
tube. Do not shake.
27) Stop the reaction by removing the staining solution and washing 4 times for 10 min
in 1 x PBST containing 10 mM EDTA.
28) Mount on glass slides in 50-80 μl Glycerol. Seal with nail polish.
2.2.14 EdU staining

EdU staining was performed according to manufacturer’s recommendations from manual with Click-iT® EdU Alexa Fluor® 488 HCS Assay (Invitrogen, cat no. C10351):

1) Prepare a 2 x working solution of EdU (Component A) from a 100 mM stock solution. Add 1:1 ratio of the 2 x EdU working solution to each tube, resulting in a 1 x EdU (10 μM) final concentration.

2) Incubate animals for 50 min in the 1 x EdU solution at RT.

3) Remove solution, wash once with fresh seawater.

4) *Fix animals in 4% formaldehyde in PBS for 20 min at room temperature, rocking.
   * If EdU staining is performed after in situ hybridisation, fix animals according to in situ protocol and proceed EdU staining with step 6) after colour reaction of in situ protocol.

5) Remove fixative and wash animals 3 times for 5 min with PBS.

6) Remove solution and wash twice with 3 % BSA/ PBS.

7) Remove solution and wash in 0.1 % Triton® X-100 in PBS for 20 min at RT.

8) Prepare 1x Click-iT® EdU buffer additive by diluting the 10 X solution 1:10 in deionised water.
   Then prepare Click-iT® reaction cocktail as following:
   430 μl Click-iT® 1 X reaction buffer
   20 μl CuSO₄ (Component H)
   1.2 μl Alexa Fluor® 488 azide (prepared according manual from component B)
   50 μl Click-iT® EdU buffer additive
   --------------------------
   500 μl total volume

9) Remove the permeabilisation buffer and wash animals twice with 3 % BSA/ PBS.

10) Remove wash solution and add 100 μl of Click-iT® reaction cocktail for reaction tubes or 500 μl for small petri-dishes. Incubate for 30 min at RT, in the dark.

11) Remove the reaction cocktail and wash animals twice for 20 min with 3 % BSA/ PBS.

12) Perform antibody or DAPI labelling of samples, if required.
2.2.15 May-Grünwald/ Giemsa staining

For cytological examination of I-cells a staining was performed according to following protocol:

1) Fix animals over night in Lav dovsky's fixative
2) Wash with Sörensen's buffer (pH 7) containing 1% Triton-X100 for 1 h.
3) Remove liquid and stain with May-Grünwald solution undiluted for 1 min.
4) Remove liquid and stain with May-Grünwald solution diluted in a 1:1 ratio with water for 2 min.
5) Remove liquid and wash four times for 1 min with water.
6) Remove liquid and stain 7 min with Giemsa solution diluted in a 1:9 ratio with water.
7) Remove liquid and wash four times for 2 min with water.
8) Mount in 90% Glycerol.
2.3 Pharmacological Wnt deregulation

To observe the role of Wnt deregulation three to five day old larva were induced to metamorphose according to protocol 2.1.2. After completion of metamorphosis the primary polyps were fed twice before treatments. Dirt particles were washed away as much as possible by repeatedly pipetting and seawater was replaced several times.

2.3.1 Wnt activation

For Wnt activation multiple chemicals are available, interfering at different points of Wnt signalling. In this study a chemical called azakenpaullone was used. This chemical blocks GSK3, belonging to the β-catenin destruction complex and thereby prevents the phosphorylation of β-catenin. As β-catenin is produced constantly and is the mediator of canonical Wnt signalling, blocking of GSK3 leads to increased Wnt signalling. A stock solution of azakenpaullone was prepared in DMSO at a concentration of 5 mM and stored at -20 °C.

After initial experiments using different azakenpaullone treatments (1 µM/ 2 µM/ 5 µM), experiments were carried out at a final concentration of 1 µM. Treatments were carried out as followed:

1) Induce larva to metamorphose (protocol 2.1.2) and place on glass cover slips for growth.
2) Feed animals twice post metamorphosis, 24 and 48 h.
3) Start of treatments: dilute stock solution of azakenpaullone with DMSO to a final concentration of 1 mM (1µl azakenpaullone stock solution to 4 µl of DMSO) and from this dilute further to a final concentration of 1 µM with seawater. Prepare also a control containing the same amount of DMSO as treatments.
4) Discard all seawater from young colony and replace directly with azakenpaullone dilution or DMSO control.
5) Incubate treatments for 18 h in the dark at 18°C.
6) Wash treated animals three times with fresh seawater.
7) Keep animals in seawater for either: 0 days/ 1day / 2days/ 3 days.
8) Incubate animals in either EdU according to protocol 2.3.2.
9) Wash three times with fresh seawater, then fix treated animals according to fixing steps of protocol 2.2.13 for in situ hybridisation or according to protocol 2.3.2 for direct EdU staining.

2.3.2 Wnt inhibition

To study the effect of Wnt inhibition on young colonies a chemical called XAV939 (Sigma, cat. no. X3004-5MG) was tested. XAV939 is a chemical that inhibits Tankyrase protein (Huang et al. 2009). This protein is responsible for Axin inhibition and therefore treatments result in an excess of Axin protein within cells. As Axin is a limiting factor during Wnt signalling and is necessary for the β-catenin destruction complex, even in the presence of Wnt ligand, which normally translocates Axin away from the destruction complex, there is plenty of axin protein left in the cell to preserve the destruction complex functional.
Stock solution of XAV939 was prepared with DMSO at a concentration of 16 mM/ml. Tested treatments ranged between 1 µM and 20 µM of XAV939 final concentration and treatment duration was varied between three h and 18 h.

1) Use either embryos from the onset of cell division, or metamorphosis induced larva (protocol 2.1.2)
2) Start of treatments: dilute stock solution of XAV939 with seawater to desired final concentration. Prepare also a control containing the same amount of DMSO in seawater as treatments.
3) Discard all seawater from animals and replace directly with XAV939 dilution or DMSO control.
4) Incubate treatments for 18 h in the dark at 18°C.
5) Wash treated animals three times with fresh seawater.
2.4 Flow cytometry

Flow cytometry (FC) is a method that counts and examines particles, such as cells. Particles are analysed by laser and grouped according to parameters such as size, cell granularity (complexity), as well as fluorescent properties can all be used. The latter also allows identifying cell surface markers bound to (fluorescent) antibodies. FC is well established for mammalian cells (e.g. blood cells), but has hardly been used in marine invertebrates. For example, components of a blood cell suspension grouped by size and granularity, allowing the different cell types to be indentified and counted.

FC would open up a new dimension in Hydractinia cell biology, because the entire animal can be dissociated to single cells and analysed by FC.

2.4.1 Dissociation protocol and sample preparation

Young colonies (~ three days post metamorphosis) have been incubated in 0.5 % pronase in seawater for three to four h at 18°C. This resulted in almost complete dissociation of the entire animal into a cell suspension. The Pronase reaction was stopped by adding a final concentration of 0.1 % BSA in seawater. The suspension was filtered through a 50 µm mesh to remove remaining tissue fragments that might block the cytometer. Concentration of cell suspensions was adjusted to 1 million/ ml for FC and 5 million/ ml for fluorescent activated cell sorting (FACS).

2.4.2 Sample run/ parameters

Cell suspensions were prepared according to the protocol in chapter 2.4.1, transferred into FACS tubes and analysed on the BD FACS CantoA in the flow facilities at NCBES/ REMEDI of NUI Galway.

CantoA colour configuration:

<table>
<thead>
<tr>
<th>Filter</th>
<th>Primary Fluorochrome</th>
<th>Other Fluorochromes</th>
</tr>
</thead>
<tbody>
<tr>
<td>530/30nm</td>
<td>FITC</td>
<td>GFP, AF488</td>
</tr>
<tr>
<td>585/42nm</td>
<td>PE</td>
<td>PI</td>
</tr>
<tr>
<td>670nm LP</td>
<td>PerCP</td>
<td>PerCP-Cy5.5, Pe-Cy5.5, PI</td>
</tr>
<tr>
<td>780/60</td>
<td>PE-Cy7</td>
<td></td>
</tr>
<tr>
<td>660/20</td>
<td>APC</td>
<td>AF647</td>
</tr>
<tr>
<td>780/60</td>
<td>APC-Cy7</td>
<td></td>
</tr>
</tbody>
</table>

For standardising the instrument, size beads from Spherotech (Spherotech, cat no. PPS-6K) of the size 3.4 µm, 7.4 µm and 14.7 µm were used.
The starting parameters on the cytometer were as following:

FSC: 8 Volt
SSC: 320 Volt
FITC: 500
FSC area scale: 0.55
Threshold: 1200

Parameters were adjusted accordingly for each experiment to place the size bead of 7.4 µm at a FSC log scale of $10^4$ and the 14.7 µm bead was used to adjust the SSC value within the measureable range. All events smaller than the 3.4 µm bead were excluded from analysis as this is supposed to be background noise. All events above the FSC log scale of $10^4$ was considered to be cells.

Cell sorts were performed by staff of REMEDI (Shirley Hanley) with BD FACS Aria II

BD FACS Aria II colour configuration:

<table>
<thead>
<tr>
<th>Filter</th>
<th>Primary Fluorochrome</th>
<th>Other Fluorochromes/dyes</th>
</tr>
</thead>
<tbody>
<tr>
<td>530/30nm</td>
<td>FITC</td>
<td>AF488, GFP, CFSE</td>
</tr>
<tr>
<td>585/42nm</td>
<td>PE</td>
<td>PI, RFP</td>
</tr>
<tr>
<td>616/23nm</td>
<td>PE-Texas Red</td>
<td>PE-CF594</td>
</tr>
<tr>
<td>695/40nm</td>
<td>PerCP</td>
<td>PerCP Cy5.5, PE-C5.5, PI</td>
</tr>
<tr>
<td>780/60nm</td>
<td>PE-Cy7</td>
<td></td>
</tr>
</tbody>
</table>

Red Laser, 633nm

| 660/20nm| APC                   | AF647                          |
| 780/60nm| APC-Cy7               | APC-H7                         |

Violet Laser, 405nm / Near UV Laser 375nm (both lasers share the same optical path)

| 450/40nm| Pacific Blue          | Cascade Blue, DAPI, Hoechst,    |
|         |                       | AF405, V450                    |
| 530/30nm| AmCyan                | V500                           |
## 2.5 Oligonucleotides used in the study

Table 2.5.1 Primer sequences. All primers were solved as stock solutions of 100 pmol/µl.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
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<tbody>
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<td>spliced leader</td>
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</tr>
<tr>
<td>newsplicedleaderfwd</td>
<td>5' ACTATTCTAAGTCCCTGAGTTTAAG 3'</td>
</tr>
<tr>
<td>splinkout</td>
<td>5' CGAATCGTAACCCGTTGTAACGCAGA 3'</td>
</tr>
<tr>
<td>splinkin</td>
<td>5' TCGTACGAGAAATCGCTGTCTCTCC 3'</td>
</tr>
<tr>
<td>SMART II</td>
<td>5' AAGCAGTGGTATAACCGCAGAATG 3'</td>
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<tr>
<td>Smart UPM long</td>
<td>5' CTATACGACTATAGGCGGTACGAG CT30VN 3'</td>
</tr>
<tr>
<td>Smart UPM short</td>
<td>5' CTATACGACTATAGGCGGTACGAG CT30VN 3'</td>
</tr>
<tr>
<td>5' CDS</td>
<td>5' T30VN 3'</td>
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<tr>
<td>3' CDS</td>
<td>5' AAGCAGTGGTATAACCGCAGAATG 3'</td>
</tr>
<tr>
<td>SP6 full length</td>
<td>5' AAGCAGTGGTATAACCGCAGAATG 3'</td>
</tr>
<tr>
<td>T7 Promoter</td>
<td>5' TAAATACGACTATAGGCGGTACGAG CT30VN 3'</td>
</tr>
<tr>
<td>UFP</td>
<td>5' GTAAACGAGGCGGCTGA 3'</td>
</tr>
<tr>
<td>URP</td>
<td>5' CAGGAAACGAGCGGCGGTACGAG CT30VN 3'</td>
</tr>
<tr>
<td>Wnt1fwd1Sp6</td>
<td>5' TAGCAATTAGGTTGACACTATAGGGAATAGCTGTAATACCGCCTAAACTAC 3'</td>
</tr>
<tr>
<td>Wnt1rev1T7</td>
<td>5' GATCATATAATACGACCTATAGGGAATAGCTGTAATACCGCCTAAACTAC 3'</td>
</tr>
<tr>
<td>Wnt1fwd2Sp6</td>
<td>5' TAGCAATTAGGTTGACACTATAGGGAATAGCTGTAATACCGCCTAAACTAC 3'</td>
</tr>
<tr>
<td>Wnt1rev2T7</td>
<td>5' GATCATATAATACGACCTATAGGGAATAGCTGTAATACCGCCTAAACTAC 3'</td>
</tr>
<tr>
<td>Wnt1fwd1RACE</td>
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<tr>
<td>Wnt1rev1RACE</td>
<td>5' GATCATATAATACGACCTATAGGGAATAGCTGTAATACCGCCTAAACTAC 3'</td>
</tr>
<tr>
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<td>5' GATCATATAATACGACCTATAGGGAATAGCTGTAATACCGCCTAAACTAC 3'</td>
</tr>
<tr>
<td>Wnt2T7 rev1</td>
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</tr>
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<td>Wnt2nest1rev</td>
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<tr>
<td>Wnt2 fwd3</td>
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<tr>
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</tr>
<tr>
<td>Wnt5ainsitu-rev</td>
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</tr>
<tr>
<td>Sp8HeWnt5A.fwd</td>
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</tr>
<tr>
<td>T7HeWnt5A rev</td>
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</tr>
<tr>
<td>Wnt5BSp6 fdd1</td>
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</tr>
<tr>
<td>Wnt5B17 rev1</td>
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</tr>
<tr>
<td>Wnt5B fdd2</td>
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<td>Wnt5B fdd2</td>
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</tr>
<tr>
<td>Wnt5B fdd2</td>
<td>5' GATCATATAATACGACCTATAGGGAATAGCTGTAATACCGCCTAAACTAC 3'</td>
</tr>
</tbody>
</table>
Wnt7fwd2SP6 5' TAGCAATTAGTGACACTATAGAACCAGCTTAGTGTAAGTACTGCA 3'
Wnt7rev2T7 5' GATCATATATACGACTCATACTAGATGGGATGATTAGTTAAAGTGCAGGCGTACGTGC
Wnt7fwd1RACE 5' CCGCTTAGTGTAAGTACTGCA 3'
Wnt7rev1RACE 5' GTGATGACTGCATCTAGATGGGATGATTAGTTAAAGTGCAGGCGTACGTGC 3'
Wnt8Sp6fwd 5' TAGCAATTAGTGACACTATAGAACCAGCTTAGTGTAAGTACTGCA 3'
Wnt8T7rev 5' GATCATATATACGACTCATACTAGATGGGATGATTAGTTAAAGTGCAGGCGTACGTGC 3'
Wnt8fwd 5' CCGCTTAGTGTAAGTACTGCA 3'
Wnt8RACE 5' GTGATGACTGCATCTAGATGGGATGATTAGTTAAAGTGCAGGCGTACGTGC 3'
Wnt8 rev 5' GTGATGACTGCATCTAGATGGGATGATTAGTTAAAGTGCAGGCGTACGTGC 3'
Wnt8newSp6fwd 5' TAGCAATTAGTGACACTATAGAACCAGCTTAGTGTAAGTACTGCA 3'
Wnt8newT7rev 5' GATCATATATACGACTCATACTAGATGGGATGATTAGTTAAAGTGCAGGCGTACGTGC 3'
Wnt8new2fwd 5' CCGCTTAGTGTAAGTACTGCA 3'
Wnt8new2rev 5' GTGATGACTGCATCTAGATGGGATGATTAGTTAAAGTGCAGGCGTACGTGC 3'
Wnt8nest1fwd 5' ACTAAATCTCACAATCAAATCTCA 3'
Wnt8nest1rev 5' GTGATGACTGCATCTAGATGGGATGATTAGTTAAAGTGCAGGCGTACGTGC 3'
Wnt9fwd1Sp6 5' TAGCAATTAGTGACACTATAGAACCAGCTTAGTGTAAGTACTGCA 3'
Wnt9rev1T7 5' GATCATATATACGACTCATACTAGATGGGATGATTAGTTAAAGTGCAGGCGTACGTGC 3'
Wnt9fwd2SP6 5' TAGCAATTAGTGACACTATAGAACCAGCTTAGTGTAAGTACTGCA 3'
Wnt9fwd1RACE 5' CCGCTTAGTGTAAGTACTGCA 3'
Wnt9rev1RACE 5' GTGATGACTGCATCTAGATGGGATGATTAGTTAAAGTGCAGGCGTACGTGC 3'
Wnt11a fdd 3' GACGGACACACAAAGAAAGTTGGAC
Wnt11a rev 3' CAACTTTCTTTGTGTGTCCGTC 3'
Wnt11a fdd nested 5' GTGTTGATAGGCGCTGTTGTAAGTACGTGC
Wnt11a rev nested 5' TCGTGTCATGATCTAGTGCAGGCGTACGTGC 3'
Wnt11b fdd 5' GTGTTGATAGGCGCTGTTGTAAGTACGTGC
Wnt11b rev 5' TCGTGTCATGATCTAGTGCAGGCGTACGTGC 3'
Wnt11b fdd nested 5' GTGTTGATAGGCGCTGTTGTAAGTACGTGC
Wnt11b rev nested 5' TCGTGTCATGATCTAGTGCAGGCGTACGTGC 3'
2.6 RNA probes used for expression analysis

To study the different Wnt gene expression patterns in all life stages of *Hydractinia echinata*, cRNA probes for in situ hybridisation were generated using the primers described later in this chapter. The gene template was amplified by PCR as described in chapter 3 and with Sp6 and T7 promoter sites attached to the 5’ ends of the primers to allow direct transcription from the template DNA into RNA probe using T7 and Sp6 RNA polymerases, respectively. DIG labelled NTP mix (Roche, cat. no. 11175025910) was used to label the probes. PCR product was assessed using gel electrophoresis and bands of the proper size were cut from the gel and the DNA extracted according to protocol 2.2.7. This was followed by sequencing to confirm that the amplicon was indeed the desired Wnt fragment. Thereafter, RNA probe synthesis was performed according to protocol 2.2.12. RNA probes were analysed for size and quality on a formaldehyde denaturing gel as described in protocol 2.2.3, and spectrophotometrically for quantity. To verify specificity of the antisense probes all experiments were also performed with sense probes as control.

**Wnt1**

To study the Wnt1 expression in all life stages of *Hydractinia echinata*, RNA probes for in situ hybridisation were created using the primers “Wnt1fwd2SP6” and “Wnt1rev2T7”.

The amplified template was 355 bp long and contained the sequence from base 695 till 1050 of the nucleotide sequence of the Wnt1 gene.

**Wnt2**

In situ hybridisation probes for the *Hydractinia echinata* Wnt2 were generated to study the gene expression pattern in all life stages. Primers used to amplify DNA template are “Wnt2Sp6 fwd1” and “Wnt2T7 rev1”.

The amplified template contained 354 bp (nucleotide 844 till end) of the coding sequence and 134 bp of the untranslated 3’ region of Wnt2, giving a total length for the probe of 488 bp.
**Wnt5A**

As stated in Chapter 3.1, Wnt5A is one of the already published Wnt genes for *Hydractinia echinata* (Stumpf *et al.* 2010). These authors reported Wnt5a expression only at the early metamorphosis, but did not comment on any other life stage. I decided to investigate whether or not this ligand is expressed at any other time point than the already described one in metamorphosis induced larva. I prepared in situ hybridisation probes according to the ones used for the previous study (Stumpf *et al.* 2010). For this I amplified the same gene fragment using identical primers as in the study (“Wnt5ainsitu-fwd and Wnt5ainsitu-rev) and then cloned the fragment into the pGEM-Vector system. This fragment was then used as template for in situ hybridisation probe synthesis according to protocol 2.2.12. The amplified template was 160 bp long and contained 132 bases (1 – 132) of the 5’ end of the Wnt5A coding sequence, as well as 75 bases belonging to the cloning vector.

**Wnt5B**

Primers used to amplify DNA template were “Wnt5BSp6 fwd1” and “Wnt5BT7 rev1”. The amplified template contained 371 bp of the coding sequence of Wnt5B from base 250 till 620.

**Wnt7**

To investigate the *Hydractinia* Wnt7 homologue probes were generated to study the gene expression pattern in all life stages. Primers used to amplify DNA template were “Wnt7fwd2SP6” and “Wnt7rev2T7”. The amplified template contained 375 bp of the coding sequence of Wnt7 (bases 688 till 1042).

**Wnt8**

To study the gene expression pattern of Wnt8 in *Hydractinia*, in situ hybridisation probes were generated. Primers used to amplify DNA template were “Wnt8newSP6fwd” and “Wnt8newT7rev”. The amplified template contained 375 bp of the coding sequence of Wnt8 (bases 469 till end) and 187 bp of the untranslated 3’ region, giving a total length of 562 bp.
Wnt9/10

In *Hydractinia echinata* one Wnt9/10 ligand could be identified. *In situ* hybridisation probes targeting the *Hydractinia* Wnt9/10 sequence were created to study the gene expression pattern in all life stages. Primers used to amplify DNA template were “Wnt9rev1T7” and “Wnt9fwd2SP6”. The amplified template contained 399 bp of the coding sequence of Wnt9/10 (bases 633 till 1031).

Wnt11A

In situ hybridisation probes were generated to study the gene expression pattern in all life stages. Primers used to amplify DNA template were “Wnt11aSP6fwd” and “Wnt11a T7rev”. The amplified template contained 547 bp of the coding sequence of Wnt11A (bases 457 till 1003).

Wnt11B

In cnidarian systems such as *Hydra* and *Nematostella* also only one member of the Wnt11 subfamily was reported. In *Hydractinia echinata* a second member belonging to this subfamily was identified. Probes for *in situ* hybridisation were generated to study the gene expression pattern in all life stages. Primers used to amplify DNA template were “Wnt11rev1T7” and “Wnt11fwd2SP6”. The amplified template contained 345 bp of the coding sequence of Wnt11B (bases 709 till 1053).

Wnt16

Also a Wnt16 homologue was identified in *Hydractinia echinata*. Primers used to amplify template “Wnt16SP6fwd” and “Wnt16T7rev”. The amplified template contained 433 bp of the coding sequence of Wnt16 (bases 606 till 10.
Chapter Three – Gene cloning and sequence analysis

3 Chapter Three – Gene cloning and sequence analysis

3.1 Introduction

Wnt signalling has pivotal roles in many aspects of normal development such as body patterning, and also functions in stem cell behaviour. This signalling pathway is known to have functions in many different animals, including humans. However, studying these complex pathways in most model organisms is difficult due to the fact that pluripotent stem cells, which can differentiate into any cell type, occur in most organisms for only a very short time period during early embryonic development. The interactions between different gene regulatory pathways are very complex in higher animals, often causing changes in other pathways and thereby disguising the direct function of the pathway of interest. As the Wnt pathway is highly conserved throughout the animal kingdom, model organisms that possess pluripotent stem cells during their entire lifecycle have become very interesting study objects (Plickert et al. 2012).

Wnt signalling has been implicated in many stem cell functions. This aspect, however, remains poorly studied in cnidarians. In *Nematostella vectensis* Wnt genes belonging to all Wnt subfamilies, with the exception of Wnt9, have been identified (Kusserow et al. 2005). It was suggested in the literature that the ramification of Wnt subfamilies has occurred previous to the splitting event of the phyla Cnidaria and Bilateria (Kusserow et al. 2006; Guder et al. 2005). However, this hypothesis remains to be corroborated. Additional data from further members of the phyla Cnidaria will give valuable insight into the early evolution of the Wnt genes.

In *Hydractinia echinata*, two Wnt genes had been identified previous to this study (Plickert et al. 2006; Duffy et al. 2010; Stumpf et al. 2010). Additionally, Teo et al (2006) showed that Frizzled, one receptor of Wnt signalling, to be expressed in proliferating i-cells in the stolonal system, which are mainly stem cells. It was reported that following ectopic Wnt activation the amount of proliferating cells in the stolons significantly increase. But specific Wnt ligands acting upstream have not been found yet. Given the high numbers of identified Wnt ligands in other cnidarians, however, I hypothesised that the *Hydractinia* genome must encode additional genes which fulfil this function.
For the search of further Wnt genes an EST database was used, available through the “Hydractinia Genome Consortium”. This presented us with partial sequences for the Hydractinia Wnt ligands Wnt2, Wnt5B, Wnt8, Wnt11A and Wnt16. To obtain more of the coding sequences, RACE PCR was performed. At a later time during this study a draft genome sequence (Provided by “Hydractinia Genome Consortium”) became available. Search for further Wnt ligands commenced and resulted in additional genes encoding Wnt1, Wnt7, Wnt9/10 and Wnt11B. Nearing the end of this study a transcriptome database (Provided by “Hydractinia Genome Consortium”) became available as well, which was utilised to predict missing gene fragments of the different Wnt ligands that failed to amplify with the help of RACE PCR.

The genes characterised in this chapter, both nucleotide or amino acid sequences, are a merger of amplified and sequenced gene fragments and predictions added with the help of the transcriptome database. The identified Wnt genes were named according to the BLAST results with highest sequence similarity.

The overall goal of this part of my studies was to identify and characterise all present Wnt genes in the hydrozoan Hydractinia echinata and study their phylogeny.

1: The “Hydractinia Genome Consortium” consists of Christy Schnitzler, Andreas Baxevanis (National Human Genome Research Institute, NIH; Bethesda, Maryland), Leo W. Buss: (Yale University, New Haven), Matt Nicotra (Thomas E. Starzel Transplantation Institute, University of Pittsburgh, Pittsburgh), Paulyn Cartwright (The University of Kansas, Lawrence), Günter Plickert (Faculty of Mathematics and Natural Sciences, University of Cologne), Philipp Schiffer (Faculty of Mathematics and Natural Sciences, University of Cologne), Cathal Seoighe (School of Mathematics, Statistics and Applied Mathematics, NUI Galway) and Uri Frank (School of Natural Sciences & Regenerative Medicine Institute (REMEDI), NUI Galway)
3.2 Experimental approach

Available EST and genome databases were screened for genes of interest. Once fragments were found, gene specific primers were designed and melting temperatures predicted by pDRAW32 DNA analysis Software of AcaClone Software. DNA fragments were then amplified according to protocol 2.2.5.1 or 2.2.5.2.

To obtain 5’ prime ends of the genes of interest a gene specific reverse primer was used together with the Hydractinia “trans spliced leader” (TSL) primer. Not all animals add a TSL to the 5’ end of their mRNAs (Hastings 2005), but many hydroids do.

Amplifying of the 3’ prime end was performed with RACE PCR using a gene specific fwd primer and a primer mix matching the 3’ CDS synthesised during cDNA synthesis for RACE cDNA. For this 1 μl of “Smart UPM long” and 2 μl “Smart UPM short” were added to 244 μl nuclease free water, mixed well and 2 μl of this mixture were used in the RACE PCR as reverse primer. Universal primers used for RACE PCR were “newsplicedleader fwd”, “Smart UPM long” and “Smart UPM short”.

Amplified gene fragments were cleaned up according to protocol 2.2.8 and used for sequencing reactions described in protocol 2.2.6 using Big Dye® Terminator v3.1 Cycle Sequencing Kit (Invitrogen/life technologies™, cat no. 4458688). The labelled gene fragments were then sent to the Genomics Core Facility at the Regional Genetics Centre of the Belfast City Hospital for sequence read-out. Obtained gene sequences were then analysed for gene identity.

In order to find putative homologues for the identified Wnt ligand, the BLAST tool from the website of the National Centre for Biotechnology Information (http://blast.ncbi.nlm.nih.gov) was used. The blastx tool was utilised to search protein databases using a translated nucleotide query. Alignment of sequences was performed with the help of the online version of ClustalW2.

Finally, phylogenetic analysis was performed using all Hydractinia Wnt genes, as well as novel Wnt genes identified in other hydrozoans together with published anthozoan, scyphozoan and bilaterian Wnt protein sequences.
3.2.1 Gene amplification by RACE PCR

cDNA for all RACE PCRs was prepared as following:

1) Total RNA extraction was performed according to protocol 2.2.1.1. This already included the DNA digestion step.

2) RACE cDNA synthesis was performed according to protocol 2.2.4.2.

3.2.2 Phylogenetic analysis

Phylogenetic analysis can be conducted using a few different mathematical algorithms, many based on Bayesian inference. This formula can then be enhanced with additional algorithms such as Markov chain Monte Carlo (MCMC) or Metropolis-Hastings-Green (MHG). An alternative approach is frequentist inference, such as maximum likelihood or maximum a posteriori estimation.

For the phylogenetic analysis presented here, a LG CAT model in a maximum likelihood framework (RAxML) was used.

The acronym RAxML stands for Randomized Axelerated Maximum Likelihood software. It is based on modifications to the standard hill climbing approach. The software calculates the 100 best-scoring phylogenetic trees with probability values between 0-100. The tree with the highest overall value is accepted as most likely. Node values are displaying how many times in the 100 rebuilds of the tree same nodes are recovered. For example, if same node was generated in 95 of the 100 test trees, a node value of 95 will be yielded in the most likely tree. Compared to more dedicated calculating software, which requires more calculating capacity and time, RAxML is commonly used for phylogenetic analysis, as the accelerated algorithm delivers good quality analysis within short calculating time.

“LG” depicts amino-acid replacement matrices, which display an improvement to previously used matrices.

A model especially devised to account for site-specific features of protein evolution is “CAT”. It is generated to increase statistical fit.
All predicted *Hydractinia* Wnt proteins were aligned with all known hydrozoan, scyphozoan, anthozoan, human, annelid and fly Wnt genes. Phylogenetic trees were generated by Dr. Paulyn Cartwright (University of Kansas). The complete phylogenetic trees can be found in the appendix (9.2.1).
Chapter Three – Gene cloning and sequence analysis

3.3 Wnt1

In order to clone *Hydractinia echinata* Wnt1, I amplified the gene via PCR as described in chapter 2.2.5.1 with the help of an available genome database. The primers used for this are “Wnt1fwd1Sp6” and “Wnt1rev1T7”.

Further fragments of coding sequence were amplified by RACE PCR according to protocol 3.2, utilising primers “Wnt1fdw1RACE”, “Wnt1rev1RACE”, together with UPM primer mix (Chapter 3.2) or “newsplicedleader fwd”, respectively.

The gene fragments were then sequenced. Results confirmed most of the predicted coding sequence and are shown in Figure 9.2.2.1 (Appendix). The sequencing files of two different sequencing approaches are merged to show the confirmed nucleotide sequence, displaying good quality of the achieved sequence. The full coding sequence of *Hydractinia* Wnt1 is given in Figure 9.2.2.2. Missing nucleotides from 5’ and 3’ end were added for this from an available transcriptome database. The 1056 nucleotide coding sequence translates to a 352 aa protein (Figure 9.2.2.3), followed by a stop codon.

The majority of possible homologues identified by BLAST search belonged to the Wnt1 subfamily. The highest similarity was suggested to be with the *Hydra vulgaris* Wnt1 protein. Alignment with ClustalW (Figure. 9.2.2.4) showed that the majority of sequence is conserved between the Wnt1 protein of those two species; fully conserved single residue positions are marked in the alignment by an asterisk (*).

To examine the sequence similarity of *Hydractinia* Wnt1, alignment of Wnt1 proteins from different phyla was performed. In addition to the *Hydractinia* protein the sequences of the species *Hydra vulgaris* and *Nematostella vectensis* as cnidarian representatives, as well as *Platynereis dumerilii* and human as bilaterian representatives were chosen. Alignment of Wnt1 proteins from different phyla showed mainly sequence similarity for cysteine positioning and common motifs (Figure 3.3.1), which are important for proper gene folding (Bazan et al. 2012). Highlighted in figure 3.3.1 is the identical sequence between all used organisms in green and amino acids which occur in four out of five organisms in orange. Overall the sequence similarity seen in the alignment is rather low with roughly 22% shared in all five sequences.
(Figure 3.3.1 green highlighted) and around 33% shared in at least four sequences (Figure 3.3.1 green and orange highlighted). In example, in literature it is suggested that human WNT1 and Drosophila wingless share around 42% sequence similarity (Miller 2001).

![Figure 3.3.1: Alignment of Hydractinia echinata Wnt1 protein and Wnt1 proteins from different organisms. "*" (asterisk) indicates positions which have a single, fully conserved residue (highlighted in green). ":" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. "." (Dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix. Amino acid residues occurring in four out of five sequences are highlighted in orange.]
Alignment was also performed with different Wnt proteins belonging to other Wnt subfamilies of *Hydra* spp. Like *Hydractinia*, *Hydra* belongs into the class of Hydrozoa. Observed sequence similarity between the Wnt1 proteins of those two species is high. Therefore, presuming classification of Wnt genes in *Hydra* would be correct, the alignment between the examined *Hydractinia* protein and *Hydra* proteins belonging to different Wnt subfamilies should give an indication whether or not sequence similarity is only due to cysteine positions and common motifs important for gene folding. The resulting alignment showed very little sequence similarity (Figure 9.2.2.5) and gave further indication that the examined *Hydractinia* protein might belong into the subfamily Wnt1.

Phylogenetic analysis also supported the hypothesis that the analysed protein belongs to the Wnt1 subfamily. Analysis of hydrozoan Wnt proteins clustered all Wnt1 proteins (Figure 3.3.2), allowing the conclusion that hydrozoan Wnt1 genes evolved lineage specific. For the entire phylogenetic tree including hydrozoan Wnt proteins see figure 9.2.1.1.

![Figure 3.3.2: Magnification of the Wnt1 cluster determined in phylogenetic analysis of hydrozoan Wnt proteins (Figure 9.2.1.1). Node values support the hypothesis that these proteins are closely related.](image)

Phylogenetic analysis including additional metazoan Wnt sequences (Figure 9.2.1.2) again resulted in clustering together of the hydrozoan Wnt1 proteins. However, the metazoan Wnt1 sequences did not cluster with the hydrozoan Wnt1 proteins and resulted in low node values (Figure 9.2.1.2). This did not support the hypothesis of evolution of Wnt subfamilies in the last common ancestor of Bilateria and Cnidaria, as was suggested (Guder et al. 2006).
3.4 Wnt2

The presence of a Wnt2 ligand was predicted by an available partial EST database, there described as homologue for the *Clytia hemisphaerica* WntX1A gene. I cloned the gene according to protocol in chapter 2.2.5.1 via PCR and RACE PCR (Protocol 3.2), using the primers “Wnt2Sp6 fwd1” and “Wnt2T7 rev1”, as well as “Wnt2fwd2”, “Wnt2rev2”, “Wnt2nest1fwd”, “Wnt2nest1rev” “Wnt2 fwd3” and “Wnt2 fwd4”.

Amplified gene fragments were used for sequencing reactions described in 2.2.6. Results from the sequencing are shown in Figure 9.2.3.1 (Appendix). Consisting of two separate sequencing approaches, the screened gene fragment displayed good quality.

The full coding sequence of the Wnt2 gene is shown in figure 9.2.3.2. Missing parts of the 5’ end of the gene were predicted with the help of genome and transcriptome databases. The full coding sequence consisted of 1197 nucleotides and translated into a 399 amino acid long protein (Figure 9.2.3.3).

Putative homologues for the identified Wnt ligand were determined by BLAST search. The results did not only show similarity to the *Clytia hemisphaerica* WntX1A protein (Figure 9.2.3.4), but suggested that the examined *Hydractinia* Wnt gene belongs to the Wnt2 subfamily, as most hits did belong into this group. However, sequence similarity was rather low between chosen organisms (Figure 3.4.1). Around 22% of amino acid residues are shared between all five (Figure 3.4.1 green highlighted) and 33% shared between four out of five organisms (Figure 3.4.1 green and orange highlighted).
Figure 3.4.1: Alignment of *Hydractinia echinata* Wnt2 protein and Wnt2 proteins from different organisms. "*" (asterisk) indicates positions which have a single, fully conserved residue (highlighted in green). ":" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. "." (Dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix. Amino acid residues occurring in four out of five sequences are highlighted in orange.
Alignment was also performed with different *Hydra vulgaris* Wnt proteins (Figure 9.2.3.5), to work out whether the shared amino acids were mainly cysteine residues and common motifs for protein folding.

Close examination of the phylogenetic analysis based on hydrozoan Wnt proteins suggested again that lineage specific evolution of this Wnt subfamily is likely. All proteins thought to belong to the Wnt2 subfamily clustered together (Figure 3.4.2). However, not all bootstrap support node values were high enough to allow clear support of lineage specific ramification. RAxML framework strongly supports relationship between the two *Hydractinia* species and *Podocoryne*, as well as between the two included *Hydra* species. The node values for remaining metazoan Wnt2 protein were too low to underpin close relationship of the proteins.

The phylogenetic analysis also including anthozoan and bilaterian datasets supported the ramification found in hydrozoan proteins (Figure 9.2.1.2). Even though all Wnt2 proteins belonging to anthozoan and bilaterian representatives cluster in close distance to the hydrozoan proteins in the calculated tree, bootstrap support node values were very low (Figure 9.2.1.2, indicated by “-”). Therefore the phylogenetic analysis does not support orthologous relationship of these proteins.
3.5 Wnt5B

Another Wnt ligand suggested by the EST database was a homologue to Xenopus \textit{Wnt5B}. This was in so far surprising, as all other previously studied cnidarians possessed only one \textit{Wnt5} gene (Kusserow \textit{et al.} 2005; Lengfeld \textit{et al.} 2009; Stumpf \textit{et al.} 2010). To amplify the predicted sequence of this gene, the primers “Wnt5BSp6 fwd1” and “Wnt5BT7 rev1” were used. Both, RACE PCR and genomic walking were performed in order to obtain more of the coding sequence, but unfortunately failed to elucidate more of the coding sequence of this Wnt gene. However, sequencing results of the amplified gene fragment displayed good quality, as shown in Figure 9.2.4.1 (Appendix). The remaining coding sequence of this gene could be predicted with the help of the transcriptome database (Figure 9.2.4.2). The 1137 bases long coding sequence translated into a 379 amino acids long protein (Figure 9.2.3.3).

Resulting hits through BLAST search using the partial gene sequence suggested indeed that the cloned Wnt ligand belongs into the \textit{Wnt5} subfamily, as most hits belonged into this group, even though the results showed low similarity except for cysteine positions and motifs important for proper protein folding. As EST database annotated this gene as homologue to the \textit{Xenopus laevis} Wnt5B protein, alignment for this protein sequence with \textit{Hydractinia} Wnt5B protein is shown in figure 9.2.4.4. Additionally, alignment of the two identified Wnt5 sequences was performed. This was done to verify that the predicted \textit{Wnt5B} gene is distinct from previously published \textit{Wnt5A} (Figure 9.2.4.5). As predominantly members of the Wnt5 subfamily were represented in BLAST results, the newly identified Wnt ligand was named \textit{Wnt5B}. However, all performed alignments did not show high similarity to this \textit{Hydractinia} gene. Therefore it is also possible that this gene is a novelty of the Hydractiniidae family. Even though highest BLAST hits belonged to \textit{Wnt5A} genes, this ligand was named \textit{Wnt5B}, as it is the second member of this subfamily identified.

Alignment of sequences belonging to the Wnt5 subfamily of different organisms showed low sequence similarity (Figure 3.5.1). Only 17.5\% of amino acid residues were shared between all five sequences (Figure 3.5.1 highlighted in green) and around 32\% were shared between four out of five proteins (Figure 3.5.1 highlighted in green and orange).
Figure 3.5.1: Alignment of _Hydractinia echinata_ Wnt5B protein and Wnt5 proteins from different organisms. "*" (asterisk) indicates positions which have a single, fully conserved residue (highlighted in green). ":" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. "." (Dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix. Amino acid residues occurring in four out of five sequences are highlighted in orange.
Comparison was also performed between *Hydractinia* Wnt5B protein and members of different Wnt subfamilies belonging to *Hydra* spp. Alignments showed mainly similarity between cysteine residues and common motifs for protein folding (Figure 9.2.4.5). As similarity between different Wnt5 proteins was higher than with other Wnt subfamilies belonging to *Hydra*, the similarity as a member of Wnt5 can be assumed correct.

To verify that the newly identified *Wnt5B* gene is not a splice variant of the *Wnt5A* gene, both, the mRNA sequence as well as the amino acid sequence of the already published *Hydractinia echinata Wnt5A* ligand were examined. The newly identified *Wnt5* ligand was distinctly different (Figure 9.2.4.6).

Phylogenetic analysis also supported the idea of a second member of the Wnt5 subfamily. It even clustered far away from the Wnt5A proteins within the hydrozoan analysis (Figure 9.2.1.1). The phylogeny also included unpublished proteins from *Hydractinia symbiolongicarpus* and *Podocoryne carnea*, which also possess a second member of the Wnt5 subfamily, clustering closely with the *Hydractinia echinata* protein (Figure 3.5.2). The occurrence of a second Wnt5 member in *Hydractinia symbiolongicarpus* and *Podocoryne carnea*, but not in other hydrozoans also supports the scenario of a lineage specific duplication event within the Hydractiniidae.

Phylogeny additionally based on bilaterian Wnt proteins also suggested no close relationship between Wnt5A group and Wnt5B in hydrozoans. In contrast to bilaterian Wnt5A and Wnt5B, which cluster together and therefore suggested evolution within the group towards a second member, the hydrozoan Wnt5B could have evolved through a lineage specific duplication event.
3.6 Wnt7

Cnidarian Wnt7 ligands were previously described both in Hydra (Lengfeld et al. 2009) and Nematostella (Kusserow et al. 2005). I identified a homologue in Hydractinia with the help of a genome database. To amplify this gene by PCR reaction, I used the primers “Wnt7fwd1Sp6” and “Wnt7rev1T7”. For RACE PCR primers “Wnt7fwd1RACE” and “Wnt7rev1RACE” were utilised.

The amplified gene fragments were then sequenced. Several approaches are merged to show the whole confirmed sequence of the gene fragment (Figure 9.2.5.1). Quality of the sequencing was good. However, RACE PCR failed to obtain the full coding sequence and the missing sequence was predicted with the help of a transcriptome database. Full coding sequence of Hydractinia Wnt7 gene is given in Figure 9.2.5.2. The 1071 bases encoded into a 357 amino acid long protein (Figure 9.2.5.3).

BLAST search for this sequence resulted mainly in hits for the Wnt7 subfamily, including the Hydra vulgaris Wnt7 protein. Alignment between Hydra and Hydractinia ligand showed 42% similarity between the proteins (Figure 9.2.5.4, asterisk). In addition a few proteins belonging to the Wnt2 subfamily were suggested as possible homologues.

Alignment with Wnt7 proteins from other organisms showed low degree of conservation. Comparison between sequences from Nematostella, Hydra, human and Platynereis showed 17% sequence similarity between all five (Figure 3.6.1, highlighted in green) and 25% between four out of five sequences (Figure 3.6.1, highlighted in green and orange). The low degree of conservation was not entirely surprising, as BLAST search results rather suggested Nematostella Wnt2 to be a possible homologue to the Hydractinia protein. However, alignment of those two proteins showed only slightly higher degree of similarity (data not shown) and therefore was dismissed as possible homologue. I also performed alignment with human WNT2B protein (Data not shown), which was also suggested by BLAST search. As with the Nematostella Wnt2 protein, conservation was limited to cysteine positions and few common motifs important for protein folding.
Alignment between the *Hydractinia* Wnt7 protein and Wnt proteins belonging to other subfamilies of *Hydra* showed even lower degree of similarity (Figure 9.2.5.5). Here nearly exclusively cysteine position and few common shared motifs were conserved.

Figure 3.6.1: Alignment of *Hydractinia echinata* Wnt7 protein and Wnt7 proteins from different organisms. “*” (asterisk) indicates positions which have a single, fully conserved residue (highlighted in green). “:” (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. “.” (Dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix. Amino acid residues occurring in four out of five sequences are highlighted in orange.
Close examination of the phylogenetic analysis supported the findings of the protein alignment. While bilaterian and anthozoan Wnt7 proteins cluster together with strong support (Figure 9.2.1.2), hydrozoan Wnt7 proteins appear to be very divergent from those proteins. However, all hydrozoan Wnt7 proteins cluster with strong support, displaying high node values in the phylogenetic analysis (Figure 3.6.2).

These findings point again towards a lineage specific evolution of hydrozoan Wnt7 genes, but do not support an evolution of the Wnt7 subfamily prior to the split of Bilateria and Cnidaria.

Figure 3.6.2: Magnification of the Wnt7 cluster determined in phylogenetic analysis of hydrozoan Wnt proteins (Figure 9.2.1.1).
3.7 Wnt8

The presence of members of the Wnt8 subfamily was reported in *Nematostella vectensis* and *Hydra vulgaris* previously (Kusserow *et al*. 2005; Philipp *et al*. 2009). Therefore it was not surprising that a Wnt8 homologue was also present in *Hydractinia echinata*. Primers were generated (“Wnt8newSP6fwd” and “Wnt8newT7rev”) according to a fragment for this gene given by a partial EST database and amplified via PCR and by RACE PCR (“Wnt8 fwd”, “Wnt8 rev”, “Wnt8new2fwd”, “Wnt8new2rev”, “Wnt8nest1fwd” and “Wnt8nest1rev”). Amplified gene fragments were then sequenced. Results of this are shown in figure 9.2.6.1 and consist of 2 different sequencing approaches, displaying high quality of the sequencing reaction. Only few amino acids were missing from 5’ and 3’ end of the protein. The remaining sequence was added with the help of the transcriptome database. Full coding sequence is given in Figure 9.2.6.2. The 1023 bases encode into a 341 amino acid long protein (Figure 9.2.6.3).

BLAST search for the identified sequence resulted in hits for the Wnt8 subfamily, verifying the classification of the original partial EST database. The Wnt8 gene of *Hydra vulgaris* showed highest degree of similarity in alignment (Figure 9.2.6.4) with a conservation of 39%.

Alignment of additional anthozoan and bilaterian Wnt8 proteins showed much lower degree of conservation (Figure 3.7.1). Only 10% amino acid sequence similarity was detected between all (Figure 3.7.1, highlighted in green) and 22% in four out of five sequences (Figure 3.7.1, highlighted in green and orange).
Figure 3.7.1: Alignment of *Hydractinia echinata* Wnt8 protein and Wnt8 proteins from different organisms. "*" (asterisk) indicates positions which have a single, fully conserved residue (highlighted in green). ":" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. "." (Dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix. Amino acid residues occurring in four out of five sequences are highlighted in orange.

To further confirm that the identified similarity was not only due to cysteine position and shared folding motifs, alignment was also performed for several different *Hydra* Wnt proteins. As displayed in figure 9.2.6.5, similarity to proteins belonging to other Wnt proteins...
subfamilies was even lower as similarity of the same subfamily in different organisms (Figure 3.7.1).

Phylogenetic analysis once more supported the findings of protein alignment. Wnt8 proteins of Hydrozoa, Anthozoa and bilaterians cluster with low statistical support (Figure 9.2.1.2), consistent with hypothesis of a lineage specific evolution of this Wnt subfamily.

In contrast, the majority of hydrozoan Wnt8 proteins clustered with strong statistical support, indicating a lineage specific ramification (Figure 3.7.2). However, node values for Wnt proteins in *Hydra*, which could not be clearly classified between Wnt subfamilies 8 and 11, was rather low (Figure 2.7.2).

Figure 3.7.2: Magnification of the Wnt8 cluster determined in phylogenetic analysis of hydrozoan Wnt proteins (Figure 9.2.1.1).
3.8 Wnt9/10

Screening of a preliminary genome database for additional Wnt genes suggested the presence of a Wnt 9/10 homologue. Similar to the Hydra homologue it was not possible to clearly assign it to either subfamily 9 or 10. To amplify the predicted gene fragment via PCR, the primers “Wnt9fwd1Sp6” and “Wnt9rev1T7” were used. Also RACE PCR was performed to obtain more of the coding sequence (Utilising primers “Wnt9fwd1RACE” and “Wnt9rev1RACE”).

Amplified gene fragments were then sequenced. Results of this are shown in Figure 9.2.7.1 (Appendix) and consist of two sequencing approaches, verifying good quality of the sequencing reaction. Missing parts of the gene could be predicted with the help of a transcriptome database, giving a full coding sequence of 1035 bases (Figure 9.2.7.2). This sequence encodes into a 345 amino acid long protein (Figure 9.2.7.3).

BLAST search using the predicted amino acid sequence showed highest similarity to the Hydra vulgaris Wnt9/10b ligand, which was chosen for alignment shown in figure 9.2.7.4. A sequence similarity of 42% between these two proteins was observed. However, when alignment was performed including also representatives from Anthozoa and Bilateria, sequence similarity between all sequences dropped to 14% (Figure 3.8.1, highlighted in green) and to 20% between four out of five sequences (Figure 3.8.1, highlighted in green and orange). It is important to note, that no BLAST hit for a Nematostella protein was suggested during the search: This is not surprising, as no members of the Wnt9 subfamily are reported for this animal. Exclusively cysteine position and common binding motifs for Wnt proteins were conserved between human and Hydractinia sequence.

To scrutinise similarity of the Hydractinia gene to the Wnt9/10 genes described in Hydra and exclude errors in classification, alignment was also performed with Hydra Wnt proteins of other subfamilies (Figure 9.2.7.5). The alignment showed very low degree of similarity (Figure 9.2.7.5, highlighted in green and orange). Mostly cases of cysteine position conservation and a few common motifs important for protein folding were shared between the compared sequences. Therefore the classification as a homologue of the Hydra Wnt9/10 protein is assumed to be correct.
The findings from BLAST search and alignment with other Wnt9 or Wnt10 proteins lead to the hypothesis that these genes did not evolve from one shared gene in the last common ancestor of Cnidaria and Bilateria.

![Alignment of Hydractinia echinata Wnt9/10 protein and Wnt9 or Wnt10 proteins from different organisms. “*” (asterisk) indicates positions which have a single, fully conserved residue (highlighted in green). “:” (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. “.” (Dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix. Amino acid residues occurring in four out of five sequences are highlighted in orange.](image)
Phylogenetic analysis supported the findings of the alignments. Hydrozoan Wnt9/10 proteins clustered with high support (Figure 3.8.2 and figure 9.2.1.2). Results suggested a close relationship of these proteins. In contrast, bilaterian Wnt9 or Wnt10 proteins, and also Nematostella Wnt9, did not cluster with the hydrozoan proteins (Figure 9.2.1.2). Therefore a close relationship to Wnt9 or Wnt10 proteins was not supported.

Figure 3.8.2: Magnification of the Wnt9/10 cluster determined in phylogenetic analysis of hydrozoan Wnt proteins (Figure 9.2.1.1).
3.9 Wnt11A

Another Wnt ligand suggested by the partial EST database was homologous to the *Clytia hemisphaerica* WntX3 gene. As this gene is published as unclassified, naming of this gene was changed after BLAST results suggested it to be a member of the *Wnt11* subfamily to *Hydractinia echinata* Wnt11A. To amplify the coding sequence the primers “Wnt11a SP6fwd” and “Wnt11a T7rev” were used for PCR and for RACE PCR primers “Wnt11a fwd”, “Wnt11a rev”, “Wnt11a fwd nested”, “Wnt11a rev nested”, “Wnt11a fwd2” and “Wnt11a rev2” were utilised.

All amplified fragments were sequenced and this resulted in the achievement of the full coding sequence of the gene. Results of the sequencing are shown in Figure 9.2.9.1 (Appendix) and consist of three separate sequencing approaches, all displaying good quality of the sequencing reactions. The confirmed 1083 bases sequence (Figure 9.2.8.2) translated into a 361 amino acid long protein (Figure 9.2.8.3). Data from the transcriptome confirmed that the obtained sequence represent the full coding sequence for this gene.

As mentioned before, the suggested gene was identified in the EST as homologue for the unclassified WntX3 gene of *Clytia hemisphaerica*. BLAST search confirmed this annotation (similarity of 30%; data not shown), but most hits belonged to the *Wnt11* subfamily, including the *Hydra vulgaris* Wnt11 gene. Alignment of these two genes showed similarities of 28% (Figure 9.2.8.4). Due to a second *Hydractinia* putative Wnt11 subfamily member (see below) this gene was named Wnt11A.

Alignment was also performed including Wnt11 proteins of three other organisms (*Nematostella, Platynereis* and human; Figure 3.9.1). Similarity of these proteins was rather low. Only 12% of amino acid residues were shared between all five organisms (Figure 3.9.1, highlighted in green) and 22% shared between four out of five organisms (Figure 3.9.1, highlighted in green and orange). With such low sequence similarity it was not surprising that BLAST search also suggested a few other Wnt proteins, belonging to other subfamilies. One of them was *Nematostella* Wnt4. However, alignment with the *Hydractinia* protein did not result in a considerable increase of sequence similarity (data not shown). The low degree of sequence similarity seen in alignments did not support the idea of shared evolution of the Wnt11 subfamily.
Figure 3.9.1: Alignment of *Hydractinia echinata* Wnt11A protein and Wnt11A proteins from different organisms. "*" (asterisk) indicates positions which have a single, fully conserved residue (highlighted in green). ":" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. "." (Dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix. Amino acid residues occurring in four out of five sequences are highlighted in orange.
To verify classification as a member of the Wnt11 subfamily and exclude the possibility that sequence similarity was only due to conservation of cysteine positioning and shared motifs important for folding of the proteins, alignment was also performed with *Hydra* proteins belonging into different Wnt subfamilies (Figure 9.2.8.5). The alignment showed even lower degree of sequence similarity; strengthen the hypothesis of classification as a member of the Wnt11 subfamily.

Likewise, the phylogenetic analysis of bilaterian and cnidarian Wnt proteins (Figure 9.2.1.2) did not support the hypothesis stated in literature, which suggests Wnt ramification prior to splitting of Cnidaria and Bilateria. While all cnidarian Wnt11 proteins clustered well and yielded high node values, the node value of ramification with bilaterian Wnt11 protein was too low to support close relationship to their cnidarian counterparts (Figure 9.2.1.2). Interestingly, not only the hydrozoan Wnt11A proteins (Figure 3.9.2), but also the proteins of other cnidarians and hydrozoan Wnt11B proteins yielded very high node values (Figure 9.2.1.2), suggesting not only close relationship of hydrozoan Wnt11 genes to each other, but also anthozoan and scyphozoan Wnt11 to be closely related.

![Figure 3.9.2: Magnification of the Wnt11A cluster determined in phylogenetic analysis of hydrozoan Wnt proteins (Figure 9.2.1.1).](image-url)
3.10 Wnt11B

As mentioned above, another member of the Wnt11 subfamily was discovered through screening of a preliminary genome database. In most cnidarians only one member of the Wnt 11 subfamily has been discovered so far. This could mean that independent gene duplication has occurred in the Hydractiniidae lineage. To amplify the suggested gene sequence, the primers “Wnt11b fwd1Sp6” and “Wnt11b rev1T7” were used for PCR and primers “Wnt11b fwd1RACE” and “Wnt11b rev1RACE” for RACE PCR in combination with universal primers (Chapter 3.2).

Sequencing of all achieved gene fragments verified the predicted nucleotide sequence of the fragment. Results of this are shown in Figure 9.2.9.1 (Appendix) and consist of a merger of two different sequencing approaches, displaying good quality of the amplified gene fragment. Still missing gene fragments could be predicted from the transcriptome database. Full coding sequence of the Hydractinia Wnt11B gene is shown in Figure 9.2.9.2. The 1080 bases encoded for a 360 amino acid long protein (Figure 9.2.9.3).

In order to find homologues for the identified Wnt ligand, BLAST was performed. The majority of the hits clustered within the Wnt11 subfamily. For an alignment the Hydra vulgaris Wnt11 amino acid sequence was used (Figure 9.2.9.4), showing high similarity between these two genes (42%). The higher sequence similarity between Hydra vulgaris Wnt11 and Hydractinia Wnt11B suggested that this might be the homologue to the Hydra protein. Hydractinia Wnt11A could therefore have evolved within the Hydractiniidae. To exclude the possibility that the two identified Wnt11 proteins are only splicing variants, additional alignment was performed between the nucleotide (data not shown) and protein sequence (Figure 9.2.9.5). Alignment of the two identified Hydractinia Wnt11 ligands showed that the transcripts are indeed distinct from each other and not splice variants of the same gene.

Even though many hits of the BLAST search belonged into the Wnt11 subfamily, alignment with sequences from Nematostella, human and Platynereis showed low degrees of similarity (Figure 3.10.1). Sequence similarity accounted for 12% shared between all five organisms (Figure 3.10.1, highlighted in green) and 23% shared between four out of five organisms (Figure 3.10.1, highlighted in green and orange). This finding was in so far not surprising, as BLAST search also suggested human
WNT4 as possible homologue. However, none of the putative vertebrate homologues displayed strong support in BLAST search. Alignment of WNT4 with the *Hydractinia* Wnt11B protein resulted in low degree of similarity (data not shown).

The low sequence similarity suggested again, that *Hydractinia* Wnt proteins are very different from Wnt proteins in other metazoans making it difficult to establish homologues.
Figure 3.10.1: Alignment of *Hydractinia echinata* Wnt11B protein and Wnt11B proteins from different organisms. "*" (asterisk) indicates positions which have a single, fully conserved residue (highlighted in green). ":" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. "." (Dot): indicates conservation between groups of weakly similar properties - scoring =< 0.5 in the Gonnet PAM 250 matrix. Amino acid residues occurring in four out of five sequences are highlighted in orange.
To validate that similarity between Wnt11 proteins of different organisms is not only due to conservation of cysteine positions and shared folding motifs, alignment was also performed with different Wnt proteins of *Hydra* (Figure 9.2.9.6). Comparison of *Hydractinia* Wnt11 with *Hydra* Wnt genes resulted in very low degree of sequence similarity, corroborating that seen similarity between Wnt11 proteins is not restricted to common features of Wnt proteins. As similarity to Wnt proteins of other Wnt subfamilies was very low and BLAST identified mainly members of the Wnt11 subfamily as putative homologues, the *Hydractinia* gene was named *Wnt11*.

To further investigate the relationship of Wnt11 proteins in hydrozoans, as well as cnidarian Wnt11 proteins to their bilaterian counterparts, phylogenetic analysis was performed. In both cases hydrozoan Wnt11A and Wnt11B proteins close relationship was strongly supported. Node values corroborated the hypothesis that this subfamily possibly evolved within the last common ancestor of hydrozoans (Figure 3.10.2 and figure 9.2.1.2).

However, establishment of the Wnt11 subfamily prior to the split between Bilateria and Cnidaria was not supported by phylogenetic analysis (Figure 9.2.1.2, node values below 70). Therefore it is possible that cnidarian *Wnt11* genes are misnamed. In contrast, the phylogenetic analysis did support the possibility of Wnt11 subfamily evolution prior to the split between Anthozoa and Hydrozoa, as node values between Cnidarians were above 70 (Figure 9.2.1.2).

Figure 3.10.2: Magnification of the Wnt11B cluster determined in phylogenetic analysis of hydrozoan Wnt proteins (Figure 9.2.1.1).
3.11 Wnt16

The presence of this Wnt ligand was predicted by an EST, as a putative homologue for the unclassified WntX1B ligand of Hydra magnipapillata. BLAST results suggested that the ligand belongs into the subfamily of Wnt16. To amplify the predicted gene sequence, primers “Wnt16 SP6fwd” and “Wnt16 T7rev” were used for PCR and primers “Wnt16 fwd”, “Wnt16 rev”, “Wnt16 fwd nested”, “Wnt16 rev nested”, “Wnt16 fwd3” and “Wnt16 rev3” for RACE PCR. The remaining parts of the coding sequence were predicted with the help of the transcriptome database.

Following amplification, gene fragments were sequenced. Results are shown in Figure 9.2.10.1 (Appendix), consisting of a merger of two different sequencing approaches, displaying good quality of the sequencing reaction. However, not the entire coding sequence was amplified and sequenced. The missing nucleotides could be predicted with the help of a transcriptome database (Figure 9.2.10.2). The 1065 bases long nucleotide sequence translated into a 355 amino acid long protein (Figure 9.2.10.3).

To classify the identified Wnt ligand BLAST was performed. The results showed highest similarity to the Wnt16 protein of Hydra vulgaris, therefore the Hydractinia ligand was named Wnt16. Alignment is given in Figure 9.2.10.4, displaying high degrees of similarity between these two proteins (45%). Further BLAST hits belonged predominantly in the Wnt4 subfamily. This also included a predicted partial sequence of a Hydra magnipapillata Wnt4 protein, which has a close to 100% identical protein sequence to the Hydra vulgaris Wnt16 protein (data not shown).

Comparison of Hydractinia Wnt16 protein to cnidarian and bilaterian (Hydra, Nematostella, human and Platynereis) Wnt16 was performed by alignment. The resulting sequence similarity was relatively low with 21% between all four organisms (Figure 3.11.1, highlighted in green) and 35% between three out of four organisms (Figure 3.11.1, highlighted in green and orange). Alignment was also performed between Hydractinia Wnt16 and human WNT4 or WNT16 (data not shown), as the human WNT4 protein was suggested by BLAST search as possible homologue. However, the two different human Wnt proteins did not show significant differences in sequence similarity when aligned with the Hydractinia Wnt16 protein, suggesting again that hydrozoan Wnt genes are very divergent from their bilaterian counterparts.
Nevertheless, the examined *Hydractinia* gene was named *Wnt16*, as similarity to *Hydra* *Wnt16* was high. In contrast, when the *Hydractinia* *Wnt16* protein was compared with *Hydra* *Wnt* genes belonging to different subfamilies, sequence similarity was rather low.
and mainly motifs important for protein folding were conserved between the sequences (Figure 9.2.10.5).

The phylogenetic analysis also underpinned the findings from the alignment. While hydrozoan Wnt16 proteins clustered with strong support (Figure 3.11.2), anthozoan and bilaterian Wnt16 proteins did not cluster with hydrozoan Wnt16 proteins (Figure 9.2.1.2). In fact, phylogenetic analysis resulted in closer ramification to anthozoan Wnt4 protein. However, node values did not suggest a relationship of those proteins either.

![Figure 3.11.2: Magnification of the Wnt16 cluster determined in phylogenetic analysis of hydrozoan Wnt proteins (Figure 9.2.1.1).](image-url)
3.12 Summary

The Wnt signalling pathway is a well conserved gene regulation system that is present throughout the animal kingdom. Previous studies had shown that most Wnt subfamilies were already present in cnidarian species (Kusserow et al. 2005). In the cnidarian Hydractinia echinata only 2 Wnt ligands had been described so far, Wnt3 and Wnt5A. During the present study 9 novel Wnt ligands for this species could be identified, provisionally belonging to 7 additional subfamilies. Based on BLAST hits the new Wnt ligands were named Hydractinia echinata Wnt1, Wnt2, Wnt5B, Wnt7, Wnt8, Wnt9/10, Wnt11a, Wnt11b and Wnt16.

The full coding sequences of these Wnt genes were obtained during this study and in situ hybridisation probes were prepared to study their expression pattern in all life stages, as described in chapter 4.

Phylogenetic analysis of the identified Wnt genes in Hydractinia was performed by Dr. Paulyn Cartwright including Wnt sequences from other hydrozoans, anthozoans and bilaterians. The Hydractinia genes clustered well with their hydrozoan homologues, but relationships with other cnidarian and bilaterian genes could not be established with confidence.
Chapter Four – Gene expression analysis

4 Chapter Four – Gene expression analysis

4.1 Introduction

Each organism needs to be able to regulate gene expression; this is the basis for cell differentiation, morphogenesis and adaptability. In diploid animals all somatic cells possess two copies of the whole genome, while gametes, which are haploid, possess only one copy. Each copy is encoding all genes existing on the DNA, but only a fraction will be expressed in each cell at any given time according to its type and activity. Protein coding genes are expressed through transcription and translation (Figure 4.1.1) (Krause 1995). Other genes, such as ribosomal RNA genes, are transcribed but not translated, as the RNA itself is the biologically active molecule (Keene 2007; Ghildiyal & Zamore 2009). In order to specify and maintain cell fate, the expression of genes can be regulated during any stage of the process.

The activation or repression of the promoter region for each gene defines whether or not a gene sequence can be read from DNA and transcribed into RNA. This is often depending on the presence or absence of certain transcription factors that bind to so called enhancer, insulator or silencer binding sites upstream of the gene promoter and therefore affect the transcription ability of this specific gene (Wolffe & Matzke 1999). A schematic illustration of the process of protein production is described in Figure 4.1.1. Once transcribed, the mRNA needs to be modified by splicing in order to cut out the non-coding introns, as well as 5’ and 3’ modifications, 5’ capping and 3’ polyadenylation respectively, before the mRNA is leaving the cell nucleus and can be translated into a protein (Matera et al. 2007; Glisovic et al. 2008). This pro-peptide chain then folds into the correct 3 dimensional structure according to amino acid interaction and is crucial for the proper protein function. The accumulation of misfolded proteins is thought to be a reason for several neuro-generative and other diseases (Luheshi et al. 2008). After translation a number of post-translational modifications can occur such as phosphorylation, acetylation and glycosylation (Wold 1981; Kia-Ki & Martinage 1992). These post-translational changes pose another step of regulation, for example marking the protein for degradation, in most cases by addition of ubiquitin. The protein will then be recognised by proteasomes and degraded.
Gene expression can be assessed at the mRNA or the protein level. In both cases different methods provide different types of information, e.g. temporal, spatial and quantitative. The most commonly used once are qualitative RT PCR, northern and western blotting, in situ hybridisation and immunohistochemistry (ICH). However, each method has its limitations (Kevil et al. 1997; Boenisch et al. 2001; Kurien & Scofield 2006; Nolan et al. 2006; Taylor & Levenson 2006). Generally speaking, each approach will have advantages and drawbacks and the method selected is therefore a compromise dictated by various factors.
Chapter Four – Gene expression analysis

The data presented in this chapter were obtained by in situ hybridisation. Therefore gene analysis was performed on the RNA level. Careful scrutinising of spatiotemporal Wnt expression might result in first clues of Wnt functions in Hydractinia. Studies conducted in Hydra, Clytia and Nematostella have focussed on the axis patterning role of canonical Wnt signalling. Indeed, in Nematostella, WntA,-1,-3, -5, -7, -11 and -16 are expressed in the oral tip of the head in adult polyps (Kusserow et al. 2005; Lee et al. 2006). None of the Wnt ligands are expressed before gastrulation in Nematostella. In Hydra head-restricted expression was reported for Wnt -1, -2, -3, -7, -9/10a, -9/10b, -11 and -16 (Lengfeld et al. 2009). Wnt5 and -8 are also expressed in the head, but not in the hypostome region, as the other Wnt ligands mentioned before. Instead Wnt8 expression can be observed at the base of tentacles and surrounding the evaginating tissue during bud formation (Philipp et al. 2009). In Clytia hemisphaerica Wnt3 expression is suggested to have a role in head formation (Momose & Houliston 2007). In Hydractinia, only two Wnt ligands have been characterised so far, Wnt3 and Wnt5A. Both genes were suggested to have a function in head formation (Müller et al. 2007; Duffy et al. 2010; Stumpf et al. 2010), but this was confirmed experimentally only for Wnt3 (Plickert et al. 2006; Duffy et al. 2010).

In this chapter the aim was to examine the expression pattern for newly identified Wnt genes in the cnidarian Hydractinia echinata and identify possible candidates for stem cell affecting Wnt genes.
4.2 Experimental approach

In the present study, the lack of specific antibodies against *Hydractinia* Wnt genes limited me to work at the mRNA level only.

I have chosen to use *in situ* hybridisation (ISH) to investigate the expression pattern of the newly discovered Wnt ligands in all life stages. For this the cloned genes (Chapter three) were used as templates to generate cRNA probes (see 2.6). Animals were fixed at different life stages and whole mount *in situ* hybridisation was performed with RNA probes for the different Wnt ligands according to the protocol described in 2.2.13. For each approach around 50 fixed animals were utilised and the entire experiment was repeated twice.

Sense control experiments were performed for all different life stages and resulted in no staining, indicating specificity of the probe.
4.3 Expression pattern during embryonic development

Of the nine newly identified Wnt ligands eight were expressed from the onset of cell divisions. Six of them were expressed throughout embryonic development till gastrulation stage. In contrast to the previously described Wnt3 ligand (Plickert et al. 2006; Duffy et al. 2010), which shows maternally polarised expression of the unfertilised egg, all novel Wnt ligands were expressed in a ubiquitous manner during embryonic development, with the exception of Wnt2, that showed a perinuclear expression. Wnt1 expression can be observed throughout the cells in a uniform manner (Figure 4.3.1 A). Gene expression at early cell division stages is due to maternal mRNA as zygotic gene transcription in Hydractinia does not start until late gastrulation (Eiben 1982).

\[\text{Figure 4.3.1: Wnt1 expression detected by in situ hybridisation during embryonic development. Wnt1 is uniformly expressed during cell division stages (A). Corresponding control is shown in B. Scale bars: 100 µm.}\]

In situ hybridisation using the Wnt2 specific probe resulted in staining throughout early cell cleavages. The Wnt2 ligand was present throughout the whole cells, but displayed higher expression levels around the cell nucleus in a so-called nuage (Figure 4.3.1 A). This expression pattern was maintained until gastrulation.
Chapter Four – Gene expression analysis

Figure 4.3.2: Wnt2 expression during embryonic development observed after in situ hybridisation. In four cell stage staining could be detected throughout the cells, with higher expression surrounding the nucleus in a so called nuage (A). Corresponding sense control is shown in B. Scale bars: 100 µm

The Wnt5B ligand was maternally deposited in high levels into the eggs in a uniform manner, as strong staining could be observed from time points when zygotic expression had not yet established (Figure 4.3.3 A).

Figure 4.3.3: Expression of Wnt5B during embryonic development. During cell division stages staining could be detected uniformly expressed throughout the cells (A). Corresponding sense control is shown in B. Scale bars: 200 µm.

Also the Wnt7 homologue was expressed throughout the embryo in a ubiquitous manner (Figure 4.3.4 A).
Expression of the Wnt8 ligand could be detected in early cell cleavages (Figure 4.3.5 A). The whole embryo stained after in situ hybridisation in a uniform manner, but expression dropped below detection level within few cleavages. After the 64 cell stage no more expression could be detected with this method (data not shown). This could mean that this ligand functions only in early stages, which is similar to Wnt8 restriction to early life stages in mouse and Xenopus. No staining in Hydra was observed at the base or tips of tentacles or in the ectoderm surrounding the evaginating tissue at bud formation, where it was observed in Hydra. No staining of Hydractinia Wnt8 was observed in the endoderm during larval development, where it was detected in Nematostella. This suggests that the role of Wnt8 differs in Hydractinia compared with sea anemones and Hydra.

Figure 4.3.5: Expression pattern of Wnt8 observed after in situ hybridisation. Uniformly staining could be observed throughout cells. (A) Hydractinia embryo at the transition from one to two cell stage, displaying uniform expression of the gene. (B) Sense control showing no staining. Scale bars: 100 µm.
The identified \textit{Wnt9/10} gene could be detected by \textit{in situ} hybridisation during embryonic development. Expression was observed in a uniform manner and was maintained throughout early cell cleavages (Figure 4.3.6 A).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure4.3.6.png}
\caption{Expression pattern of Wnt9/10 during embryonic development. A) Eight-cell embryo stained after \textit{in situ} in a uniform manner. B represents corresponding sense control staining. Scale bars: 100 \textmu m.}
\end{figure}

\textbf{After \textit{in situ} hybridisation experiments weak staining for the \textit{Wnt11B} ligand could be observed during early cell cleavage stages (Figure 4.3.7 A). This expression level weakened with each division, suggesting that the \textit{Wnt11B} mRNA is rapidly degrading and has a function in very early development only, but not during later embryonic stages.}
Figure 4.3.7: Wnt11B expression during embryonic development. (A) Weak expression was observed in four cell stage embryos, uniformly distributed throughout the cells. B represents corresponding sense control staining. Scale bars: 100 µm.

In contrast to the weak Wnt11B expression, embryos stained strong targeting the Wnt16 ligand (Figure 4.3.8 A). High expression levels were observed throughout embryonic development, suggesting that this Wnt ligand is not only maternally deposited, but that the embryo maintains this expression level after zygotic transcription commences.

Figure 4.3.8: Expression of Wnt16 during embryonic development. (A) Strong expression of the Wnt16 ligand could be detected throughout cell division stages in a uniform manner. B represents corresponding sense control staining. Scale bars: 200 µm.
4.4 Expression patterns at the larva stage

After about two and a half days post fertilisation the animal reaches the stage of planula larva, in which it can crawl and reach new habitats.

Expression of Wnt1, Wnt 5B, Wnt 7, Wnt 9/10 and Wnt 16 was observed by in situ hybridisation at this life stage. Also the already published Wnt3 ligand is expressed at this stage, at the posterior side of the larva (Plickert et al. 2006; Duffy et al. 2010).

In contrast to the uniform expression of Wnt1 in the embryo, mRNA of this gene could only be detected in few cells in the epidermis at the posterior side of the larva tail at this stage by in situ hybridisation (Figure 4.4.1 A and C).

Figure 4.4.1: Expression of Wnt1 detected by in situ hybridisation in planula larva. (A) Wnt1 expressing cells are restricted to the posterior tip. (C) Higher magnification of the posterior tip. Expression was restricted to very few cells in the epidermis. Corresponding control is shown in B. Scale bar: A/ B 200 µm; C 50 µm
In comparison to \textit{Wnt1}, the \textit{Wnt5B} ligand remained broadly expressed throughout the larva, but expression was weaker in the last third of the posterior side of the larva tail (Figure 4.4.2 A).

![Figure 4.4.2: Expression of \textit{Wnt5B} at the larva stage. Expression was observed in both, epi- and gastrodermis starting from the anterior side till the posterior third of the larva (A). Expression was weaker in the last third of the larval tail. Corresponding sense control is shown in B. Scale bar 200 µm.](image)

The uniform expression of \textit{Wnt7} and \textit{Wnt9/10} observed in embryos was preserved throughout the larva stage (Figure 4.4.3 A and 4.4.4 A). Both, cells of the gastrodermis and epidermis apparently were expressing these Wnt ligands.
Chapter Four – Gene expression analysis

Figure 4.4.3: Detection of *Hydractinia echinata* Wnt7 ligand by *in situ* hybridisation at larva stage. (A) During larva stage the expression appears to be uniformly distributed throughout the larva. B represents corresponding sense control. Scale bars: 200 µm.

Figure 4.4.4: Expression of *Wnt9/10* at larva stage. (A) Expression was present in a uniform manner throughout the animal. B represents corresponding sense control staining. Scale bars: 200 µm.

Expression of the *Wnt16* ligand was observed uniformly throughout the gastrodermis at the larva stage. Also the epidermis in the anterior side of the larva ostensibly stained positive, but expression was apparently excluded from the posterior side in the epidermis (Figure 4.4.5 A).

120
Figure 4.4.5: Expression pattern of Wnt16 ligand observed after in situ hybridisation. (A) In larva stage the expression pattern was excluded from the posterior side of the larva, which is the prospective head of the adult stage. B represents corresponding sense control staining. Scale bar: A/ B 100 µm.
4.5 Expression patterns after metamorphosis induction

Three days post fertilisation the larva becomes perceptive to metamorphosis inducing signals. In nature a bacterial film on the shells of hermit crabs represents this signal. In the laboratory metamorphosis can be triggered by incubation of metamorphosis-competent larva for three and a half hours of 116 mM CsCl in seawater. During metamorphosis the induced larva settles down with the anterior side on a substrate (such as glass slides), which will develop into the aboral structures of the polyp. The larva tail contracts until the animal has a teardrop shape and develops into the polyp’s oral end. Metamorphosis is completed within 24 hours and the larva has transformed into a primary polyp, consisting of a head surrounded by tentacles, a body column and aboral structures, which are called stolons.

Animals used for in situ hybridisation of this stage were incubated in CsCl, washed three times in seawater and fixed after another one and a half hours to allow tail contraction and start of gene expression for this stage. After metamorphosis induction expression of the genes \textit{Wnt1}, \textit{Wnt2} and \textit{Wnt7} could be detected by in situ hybridisation, in addition to the previously published \textit{Wnt5A} and \textit{Wnt3}.

Few cells expressing \textit{Wnt1} were observed in the epidermis at the posterior side of the larva tail, which corresponds to the future oral pole (Figure 4.4.5 A).

![Figure 4.5.1: Wnt1 expression detected by in situ hybridisation of metamorphosis induced larva. After induction of metamorphosis the expression persists in the posterior tip while the tail contracts (A). Corresponding controls are shown in B. Scale bars: 100 µm](image)
Strong staining for \textit{Wnt2} could be detected in the posterior tip of the larva tail after metamorphosis induction and weaker staining in the remaining larvae (Figure 4.5.2 A). \textit{Wnt2} ligand was absent in larvae that were not induced for metamorphosis, suggesting that this gene might have a function during the metamorphosis processes.

![Figure 4.5.2: Wnt2 expression observed in induced larva stage after \textit{in situ} hybridisation. In metamorphosis induced larva stages the expression was observed strong in the posterior larva tip and weak expression throughout the rest of the larva. Corresponding sense controls are shown in B. Scale bar: A/ B 200 µm.](image)

As previously stated in Chapter 3.1, the \textit{Wnt5A} ligand is one of the already published Wnt genes for \textit{Hydractinia echinata} (Stumpf \textit{et al.} 2010). It was discussed regarding its organiser function during metamorphosis. But as the mentioned study represents the only publication about this gene in \textit{Hydractinia} and there was no further description about this gene in any other life stage, I decided to investigate whether or not this ligand is expressed at any other time point as well. I performed \textit{in situ} hybridisation experiments for all life stages. Exclusively in the metamorphosis induced larva stage expression for the \textit{Wnt5A} gene could be confirmed (Figure 4.5.3 A). Only few cells of the epidermis showed \textit{Wnt5A} expression in metamorphosis induced larva, as previous described in the study of Stumpf \textit{et al.} (2010). \textit{In situ} hybridisation experiments for all other stages resulted in no staining. This could mean that the ligand is only present at this stage or that the expression level is too low for detection with this method in other life stages.
Chapter Four – Gene expression analysis

Figure 4.5.3: Wnt5A expression detected by in situ hybridisation of metamorphosis induced larva. Expression could be observed in the posterior tip while the tail contracts (A), as previously published in Stumpf, Will et al. (2010). Corresponding controls are shown in B. Scale bars: 100 µm

Only for the Wnt7 ligand a broad expression throughout the induced larva could be detected after metamorphosis induction (Figure 4.5.4 A). However, a slight increase of expression could be observed in gastrodermal cells at the posterior tip (Figure 4.5.4 A arrow).

Figure 4.5.4: Expression pattern found after in situ hybridisation with the Wnt7 homologue of Hydractinia echinata. (A) After metamorphosis induction the expression was present throughout the larva, with a slight increase in expression level in the posterior tip of the tail could be observed in gastrodermal cells (arrow). B represents corresponding sense control experiment. Scale bars: 100 µm.
4.6 Expression patterns in stolons

After completion of metamorphosis the larva has transformed into a primary polyp. It consists of oral structures that include mouth and tentacles, a body column and aboral structures. Aboral structures are called stolons, which are a gastro-vascular system, connecting the entire colony and distribute food particles between individuals. In the stolons also the majority of stem cell, so called interstitial cells (i-cells), can be found. However, i-cells can only be identified based on their morphology in *Hydractinia*. Therefore identification in intact and unstained tissue was difficult. Additionally, candidates of Wnt genes affecting stem cell do not have to be expressed by the stem cells themselves. As Wnt proteins are secreted and act as morphogens, expression could possibly be detected in surrounding tissue. Global Wnt activation was shown in a past study to affect stem cell amounts in the stolonal system (Teo et al. 2006). Therefore Wnt genes expressed in proximity of stem cells in the stolons are considered as possible candidates for affecting stem cell function.

Of the novel Wnt ligands *Wnt2*, *Wnt11A* and *Wnt16* were expressed in young colonies. Also the *Wnt3* is expressed at this stage in the head of the primary polyp (Duffy et al. 2010).

After completing metamorphosis, no *Wnt2* expression could be detected in 24 hours old primary polyps (data not shown). In young colonies cultured for a few days, where stolonal growth had progressed enough for stolons to branch and new polyp buds to form, strong *Wnt2* staining could be detected by *in situ* hybridisation (Figure 4.6.1). In close proximity to the primary polyp, weak expression levels in scattered cells in the epidermis (Figure 4.6.1 B arrow-heads) could be observed. Newly out growing stolons from the primary polyp base displayed strong expression levels, both epi- and gastrodermis (Figure 4.6.1 B arrow). In more distal parts of the stolon, in areas where the stolon was about to branch out or the formation of a new polyp bud had begun, strong expression of the *Wnt2* ligand was observed (Figure 4.6.1 A/ C/ D star). Both epi- and gastrodermal cells express the *Wnt2* gene.
In situ hybridisation experiments targeting the *Hydractinia* Wnt11A homologue in early development resulted in no staining, indicating that the gene is not expressed during...
cleavage or larva stages. Also during metamorphosis induction and reorganisation into the primary polyp no expression could be detected (data not shown). After few days of growth of the primary polyp, allowing the stolons to expand, strong expression could be detected in the tips of the outgrowing stolons (Figure 4.6.2). Also few cells along the stolon expressed \textit{Wnt11A} (Figure 4.6.2 A arrow head), but significantly lower than cells staining in the tip. Closer look on these cells suggested that they might be neurons, as stained cells display not only a cell body, but also neurite like elongations (Figure 4.6.2 D arrows).

Figure 4.6.2: Expression pattern observed after \textit{in situ} hybridisation targeting the \textit{Hydractinia Wnt11A} ligand. (A + B) Expression of this gene could be detected in the tips of outgrowing stolons. (D) The morphology of the stained cells suggests that neurons in the growing zone of the stolon tip are expressing the \textit{Wnt11A}, as stained cells displayed neurite like extensions (arrows). (C) Sense control experiment, verifying the specificity of the generated probe. Scale bars: 50 µm.

Throughout metamorphosis the expression level of \textit{Wnt16} stayed below detection level, but was increased again in the entire primary polyp and stolons after completion of metamorphosis (Figure 4.6.3 A/ B). Higher magnification of the stolons revealed that the entire gastrodermis expresses the \textit{Wnt16} ligand, but not the epidermis (Figure 4.6.3 B). All efforts to identify single cells that express this gene were inconclusive.
Figure 4.6.3: Wnt16 expression pattern observed in young colonies of *Hydractinia echinata*. (A) Overview of a primary polyp (out of focus) and outgrowing stolons, displaying staining throughout the gastrodermis. (B) Higher magnification of stolonal tissue illustrates that only the gastrodermis is stained. C and D represent corresponding sense control staining. Scale bar: A/ C 100 µm; B/ D 50 µm.
4.7 Expression patterns in adult feeding polyps

Adult feeding polyps cut off the colony were examined for expression of the novel Wnt ligands. These polyps provide the whole colony with food, as they catch prey with the tentacles from surrounding waters and digest it in the gastric cavity, before food particles are distributed through the stolonal system to all members of the colony. In addition to the published Wnt3 expression in the head, the genes Wnt1, Wnt2, Wnt7, Wnt9/10, Wnt11B and Wnt16 could be detected by *in situ* hybridisation at this stage.

Feeding polyps exhibited a clear staining of epidermal cells in the head area (Figure 4.7.1 A and C) after *in situ* hybridisation with the Wnt1 probe. This is consistent with the expression pattern of Wnt1 shown in other cnidarian models. It might indicate that a function of Wnt1 is conserved in head specification in cnidarians. Higher magnification of the head area revealed distinct cells in the epidermis are expressing the gene (Figure 4.7.1 C arrow heads).
Figure 4.7.1: Wnt1 expression in feeding polyps. Epidermal cells in the head area stained strongly with the antisense probe (A + C). Sense control showed no staining at all (B). Scale bar: A/ B 200 µm; C 50 µm.

In contrast to Wnt1, the expression of Wnt2 in feeding polyps was detected in the body column (Figure 4.7.2 A). Here either single cells (Figure 4.7.2. C arrows) or clusters of cells (Figure 4.7.2 C star) expressed Wnt2.
Figure 4.7.2: Wnt2 expression in feeding after *in situ* hybridisation. Expression in feeding polyps could be detected in the body column (A). Higher magnification in C revealed that single cells (arrows) or clusters of cells are stained (star). Corresponding sense control is shown in B. Some unspecific staining was observed (arrow-head). Scale bar: A/ B 200 µm; C 50 µm.

Wnt7 expression could be observed, like Wnt1, in fully grown feeding polyps in the head area, staining in a broad area (Figure 4.7.3 A), with stronger expression in scattered single cells surrounding the mouth opening (Figure 4.7.3 C). This expression pattern is consistent with Wnt7 homologues in other cnidarians, where a broad area in the head expresses this gene as well.
Wnt7 expression pattern observed after in situ hybridisation of adult feeding polyps. (A) Expression of the Wnt7 homologue could be found in a broad manner all over the mouth area. Higher magnification of the head revealed stronger expression in scattered single cells surrounding the hypostome (C). B represent corresponding sense control experiments. Scale bar: A 100 µm; B/ C 50 µm.

Wnt9/10 expression could also be detected after in situ hybridisation experiments in the head of adult feeding polyps (Figure 4.7.4). Higher magnification of scattered cells suggested that they might be neurons, due to their position and morphology (Figure 4.7.4 C), as Wnt9/10 expressing cells exhibit neurite like extensions (Figure 4.7.4 C arrows).
In adult feeding polyps expression of Wnt11B was detected in the epidermis along the body column (Figure 4.7.5 A), but higher magnification did not elucidate which cell type exactly expressed the Wnt11B ligand in this area. In the head region stronger expression levels were visible (Figure 4.7.5 C), displaying a sharp border of high expression in the hypostome to lower expression level detected throughout the animal.
Figure 4.7.5: Expression pattern observed after in situ hybridisation targeting the *Hydractinia Wnt11B* ligand. (A) In adult feeding polyps staining could be detected in the epiderm throughout the polyp. The head area surrounding the mouth opening exhibits a higher expression level. (C) Higher magnification of head. B represents corresponding sense control staining. Scale bar: A/ B 200 µm; C 50 µm.
The expression of *Wnt16* in the entire gastrodermis was temporarily very restricted. Only young polyps displayed this staining (see Figure 4.6.3), while in adult feeding polyps no expression of the gastrodermis was observed anymore. Instead expression pattern for the *Wnt16* ligand was detected in an epidermal belt of small cells around the lower parts of the body column (Figure 4.7.6 A). Higher magnification of this area revealed that small cell clusters scattered in the tissue layer express the *Wnt16* ligand (Figure 4.11.3 B and E).

**Figure 4.7.6:** *Wnt16* expression detected by *in situ* hybridisation in adult feeding polyps. (A) *Wnt16* expressing cells were observed in the lower part of the body column. Higher magnifications of this area are highlighted in B/ E show that small cell clusters express the *Wnt16* ligand. Corresponding sense control is displayed in C and D. Scale bar: A/ C 200 µm; B/ D/ E 50 µm.
4.8 Expression patterns in sexual polyps

In contrast to other medusozoa in _Hydractinia echinata_ no free swimming medusa stage is present, but it is instead reduced to a gonad, which remain attached to the colony. As sexes are separate in _Hydractinia_, either male or female sexual polyps can be found. This specialised polyps type then produces either oocytes or sperm, depending on the sex of the colony. These are then released into the surrounding water where fertilisation takes place.

As in all other life stages, _Wnt3_ is also expressed in sexual polyps in the head. Of the novel Wnt ligands _Wnt2_ and _Wnt16_ expression could be detected by _in situ_ hybridisation. Both were observed in young gonads of male sexual polyps - possibly germ cells - (Figure 4.8.1. A), but not in females.

![Figure 4.8.1: Wnt2 expression pattern in male sexual polyps after in situ hybridisation. (A) In male sexual polyps early gonads stained positive targeting Wnt2. B represents corresponding sense control. Scale bars: 50 µm.](image)

The second Wnt ligand detected in male sexual polyps was _Wnt16_ (Figure 4.8.2). Expression was observed in cells throughout young gonads (Figure 4.11.4 A and B), similar to _Wnt2_. Weak expression was also observed in more mature gonads.
Figure 4.8.2: \textit{Wnt16} expression pattern observed in male sexual polyps after \textit{in situ} hybridisation. (A and B) Strong expression was detectable by \textit{in situ} in young gonads (arrow head), as well as weak expression in more mature gonads (star). B displays a higher magnification of a young gonad with distinct cells expressing the \textit{Wnt16} gene. C and D represent corresponding sense control. Scale bar: A/ C 100 µm; B/ D 50 µm.
4.9 Summary

I performed *in situ* hybridisation experiments for all identified Wnt ligands throughout the life stages of *Hydractinia echinata*. Expression patterns observed (Figure 4.9.1) suggest that many Wnt ligands have a function in axis specification and possibly also in neurogenesis, nematogenesis and spermatogenesis.

![Figure 4.9.1: Schematic drawing of Wnt gene expression observed following in situ hybridisation. Shown are expression domains for (A) embryonic development, (B) planula larva, (C) metamorphosis induced larva, (D) primary Polyp, (E) adult feeding polyp and (F) male sexual polyp.](image-url)
Table 4.9.1: Summary of expression for all identified Wnt ligands in *Hydractinia echinata*. Wnt ligands published previous to this study are marked with a star. Shown are expression patterns for embryos, larva, metamorphosis induced larva (ind. larva), stolons, feeding polyps and sexual polyps.

<table>
<thead>
<tr>
<th>Wnt1</th>
<th>embryo</th>
<th>larva</th>
<th>Ind. larva</th>
<th>Stolons</th>
<th>Feeding polyp</th>
<th>Sexual polyp</th>
</tr>
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<tbody>
<tr>
<td>Wnt2</td>
<td>X</td>
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<td></td>
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Expression of the *Wnt1* mRNA was uniform in embryos. In larvae it became restricted to the posterior side of animal in few ectodermal cells. No expression could be observed in young colonies, but in adult feeding polyps *Wnt1* was detected in the head of the polyp. The collected data from the *Wnt1* ligand indicate that a main function of this ligand might be in defining the head area before metamorphosis induction and positional information of the head in adult stages.

*Wnt2* was expressed in most life stages of *Hydractinia*. The gene’s mRNA was detected in embryonic development, as well as in metamorphosis induced larva. After completion of metamorphosis, expression was observed in distal parts of the stolon, were either branching events or formation or a new polyp bud had begun. In feeding polyps *Wnt2* was expressed in the lower part of the body column, expressed either in single cells or cell clusters. Also in male sexual polyps this gene’s RNA could be detected in young gonads. Expression was mainly observed in areas of active growth, suggesting that this Wnt ligand might have a function in regulation of proliferation.

The *Wnt3A* ligand was not examined in depth in this study, as this was already done in previous studies (Plickert *et al.* 2006; Duffy *et al.* 2010), showing a clear function in head formation and maintenance of the positional information. Expression could be found in all life stages in the oral tip. When over-expressed or examined following global Wnt activation, ectopic head structures were identified all over the body column, expressing Wnt3A. In extreme cases, global Wnt activation lead to total oralisation.
during metamorphosis Wnt3A was, however, used as positive control in this study (data not shown).

The other previously published Wnt gene, Wnt5A, was examined for further expression other than the described expression during metamorphosis. I was able to confirm that the gene is only expressed in the posterior tip of metamorphosis induced larva and not in any other life stage. This suggests that the sole function of the Wnt5A ligand is to help establish an organisation centre during metamorphosis and prevention of apoptosis of these cells (Stumpf et al. 2010).

Surprisingly, a second Wnt5 ligand could be identified in Hydractinia echinata, which is unique for cnidarians. The expression of the second Wnt5 ligand, Wnt5B, could only be detected during embryonic development and evidently was complementary at the larva stage to the Wnt5A ligand, excluded from the posterior tip of larva prior to induction of metamorphosis.

Another identified Wnt ligand was Wnt7, which is also expressed during embryonic and larva stages, but in a ubiquitous manner. During metamorphosis induction an increase of expression level of the most posterior epidermis could be observed. After completion of metamorphosis mRNA of this gene could only be detected in adult feeding polyps, expressed in a broad area of the head.

The expression of Wnt8 was restricted only to early embryogenesis, as found in mouse and Xenopus. This is a contrast to expression patterns found in other cnidarians for this gene, as both in Hydra and Nematostella the gene is expressed in adult stages in bud formation and in the tentacle in Hydra, and in Nematostella expression was observed in the forming mesenteries at late planula and polyp stage.

Examination of the expression pattern by in situ hybridisation targeting Wnt9/10 displayed ubiquitous staining during embryonic and larva stages. After metamorphosis induction this expression could no longer be detected, until reaching adult feeding polyp stage, where scattered cell surrounding the mouth opening showed expression of Wnt9/10. Morphology and position suggests that these cells were neurons.
Wnt11A identified in *Hydractinia* is the only Wnt ligand that was not expressed during early development. Instead expression of this gene could only be detected in the tips of stolons in young colonies. Cells expressing this gene appear from their morphology to be neurons, based on neurite like elongations.

The second Wnt11 member identified, Wnt11b, after *in situ* hybridisation showed a very weak staining in the first cleavage stages, but detection was already not possible with this method after few more cell divisions. No expression could be observed later in embryonic development, larvae stages, metamorphosis or in young colonies. Only in adult feeding polyps higher expression levels could be observed. Here the staining was visible throughout the body column in the epidermis. A slightly higher level of expression was observed in the head area surrounding the mouth opening.

The last identified Wnt gene in *Hydractinia* was Wnt16. Except for metamorphosis, Wnt16 could be detected in all life stages. Embryos displayed a ubiquitous staining, while in planula larva the expression became excluded from the posterior side of the animal. After completion of metamorphosis, expression could be detected throughout the gastrodermis of primary polyps and stolons. The gastrodermal expression was temporarily restricted and in adult feeding polyps no such staining was observed anymore. Instead Wnt16 mRNA could be detected in the lower part of the body column in a belt like pattern in the epidermis. Expression of the Wnt16 gene was also observed in male sexual polyps. Here the outer layer of young gonads displayed staining after *in situ* hybridisation. The data collected about the Wnt16 gene leads to the hypothesis that this gene might have a function in the regulation of proliferation.
5  Chapter Five – Gene expression after Wnt deregulation

5.1  Introduction

It was shown in past studies, that Wnt signalling controls stem cell behaviour (Reya et al. 2003; Willert et al. 2003; Sato et al. 2004; Reya & Clevers 2005). Whether Wnt signalling supports self-renewal (Zeng & Nusse 2010; Lim et al. 2013) or differentiation in stem cells (Kim et al. 2013; Zhang et al. 2013) is not yet clear in all systems studied. In the literature, studies supporting either of the hypotheses can be found, and it appears that Wnt acts differently in different contexts.

Stem cells represent great potential for regenerative medicine (Gimble et al. 2007; Mimeault et al. 2007; Sasai 2013), as they might be utilised to replace damaged or aged cells and tissue following accidents or diseases. But to be able to do so, regulation and target-orientated differentiation needs to be deciphered and fully understood. In the past decades much effort has been undertaken to examine stem cells and investigate their capacities. However, stem cell research in higher organisms, such as humans, is subject to many ethical issues (Sandel 2004; Sugarman 2008) and technical difficulties. For example, studying the properties of stem cells can be mainly performed using cell cultures where cell behaviour is not necessarily identical as in vivo (Guilak et al. 2009). Stem cells, which are capable to form cells belonging to all three different germ layers, so called pluripotent stem cells, occur in most animals only during early embryonic development, but it has been suggested that in certain invertebrates these cells are present throughout the entire life (Plickert et al. 2012). Therefore, studying pluripotent stem cell decision-making and regulation in these systems displays great potential due to their life-long availability. Furthermore, no ethical restrictions exist for work on invertebrates.

Wnt signalling has been implicated in many stem cell functions. This aspect, however, remains poorly studied in cnidarians. In Nematostella multipotent stem cells have not been characterised (Watanabe et al. 2009). Therefore studying the properties and behaviour of pluripotent stem cells is not possible in this model organism. In contrast, Hydra possesses three different types of stem cells, including the so called interstitial cell (i-cell) (David & Murphy 1977; Bode 1996). I-cells were shown to be capable to differentiate into a few different cell types (David & Murphy 1977; Watanabe et al. 2009). However, i-cells in Hydra usually do not give rise to epithelial cell, which are
replenished from the other two stem cell types present. Therefore multipotency has been confirmed in *Hydra*, but not pluripotency.

One invertebrate that is believed to possess pluripotent stem cells throughout its life span is *Hydractinia echinata* (Müller *et al.* 2004; Künzel *et al.* 2010; Plickert *et al.* 2012). *Pln*, a critical stem cell gene, has been identified in *Hydractinia* (Millane *et al.* 2011). It is a POU domain gene homologue.

A past study has shown that these stem cells, which are mainly found in the stolonal system of the animal, express the Frizzled receptor (Teo *et al.* 2006). It was also reported that after global Wnt activation a temporal increase of proliferating cells can be observed, suggesting that stem cell behaviour is indeed affected by altered Wnt signal (Teo *et al.* 2006).

However, the two previously described Wnt genes in *Hydractinia* are not expressed in the stolons, where the majority of i-cells reside. Nine further Wnt genes are present in *Hydractinia*, as identified in chapter three. Of these genes, expression of *Wnt2, Wnt11A* and *Wnt16* was observed in proximity to stem cells (Chapter 4.6). Furthermore, Wnt genes act as morphogens. This means the ligand is secreted and can thereby affect the secreting cell and neighbouring cells. Different approaches to alter Wnt signalling are feasible, either targeting specific genes (for example microinjection of over expression constructs or RNAi treatments) or global Wnt targeting (chemical treatments).

As many Wnt ligands have pivotal roles during embryonic development and conditional expression vectors have not yet been established in *Hydractinia*, global Wnt activation or inhibition was performed. It is not yet clear which Wnt ligand is affecting stem cells in this animal. One aim of this study was to identify possible candidates of stem cell affecting Wnt ligands in *Hydractinia* by examination of expression pattern after Wnt deregulation. This experiment could show which Wnt genes possess a feedback loop. The goal was to identify Wnt genes where the expression would mimic the proliferation wave observed after global activation of canonical Wnt signalling.

For global Wnt activation a chemical compound called azakenpaullone was used. Azakenpaullone specifically inhibits the enzyme GSK3, which is an antagonist of
canonical Wnt signalling, and therefore activates the Wnt pathway, as was already established by other researchers (Teo et al. 2006).

Opposite experiments with a chemical that is inhibiting Wnt signalling in other animals (ZAV939; (Fearon 2009; Liu & He 2010)) were performed as well.

EdU staining was used to assess the effect of Wnt deregulation on proliferating cells. EdU staining was performed after treatments to examine the amount of proliferating cells. As most proliferating cells in the stolonal tissue are thought to be stem cells, staining of proliferating cells can give an indication about the amount of stem cells present after different treatments.

The overall goal of this part of the study was to reveal the spatiotemporal manner in which the proliferating cells in the stolons react to global Wnt activation. Additionally it was a goal to investigate how the expression pattern of Wnt genes themselves respond following ectopic Wnt activation and identify all possible candidates of stem cell affecting Wnt genes.
5.2 Experimental approach

For global Wnt activation three to four day old planula larvae were induced to metamorphose (Protocol 2.1.2). Per coverslip 15 induced larva were placed, allowing enough space for growth of each animal. Once metamorphosis was completed, animals were fed twice on consecutive days to ensure survival throughout the treatments. For this each feeding polyp was fed one *Artemia salina* nauplii separately. The young colonies were treated with azakenpaullone to globally activate Wnt signalling according to protocol 2.3.1. Following Wnt activation, either proliferating cells were labeled with EdU according to protocol 2.2.14 to investigate the effect of Wnt activation on cell proliferation, or animals were fixed directly to perform *in situ* hybridisation.

Consecutively to EdU labelling, the proliferating cells were stained according to manufacturer recommendation. The number of proliferating cells for all different treatments was counted in areas with the highest numbers of EdU positive cells. For this, the stolonal system of stained colonies were screened for area with highest density of EdU cells. To ensure comparability between different treatments, an equally sized area was utilised for counting cells.

As both, azakenpaullone and XAV939, were solved in DMSO, it was necessary to exclude the possibility that the solvent affected the experiment outcome. Control experiments were performed with untreated colonies (only seawater) and colonies that were incubated in DMSO at equal amount and duration utilised for Wnt deregulation treatments.

Finally, to investigate which Wnt ligands might be target of canonical Wnt signalling itself, *in situ* hybridisation was carried out following azakenpaullone treatment. Special attention was paid on expression in the stolons. Stem cell affecting Wnt genes should be expressed either by the stem cell itself, or in close proximity of stem cells as Wnt ligands diffuse only short range.

Global Wnt activation experiments were replicated twice under equal treatment conditions.
For global Wnt inhibition either developing embryos or metamorphosis induced larva were utilised. Around 20 animals were treated with XAV939 according to protocol 2.3.2 for each experiment. Preliminary experiments were performed to define treatment conditions. Treatment durations were tested for three, five, eight, 10 and 18 hours at final concentrations of 1 µM, 2 µM, 10 µM and 20 µM of XAV939 in seawater.

All preliminary Wnt inhibition experiments were carried out twice.
5.3 Wnt activation

5.3.1 EdU staining after Wnt activation

Animals for experiments were prepared as laid out in the experimental approach (Chapter 5.2) and treated with azakenpaullone at a final concentration of 1 µM/ml for 18 hours (Protocol 2.4.1). Control experiments were performed both with untreated colonies and colonies that were incubated in 1 µl DMSO per ml SW (Equal amount as in treatments). This was followed by EdU staining according to protocol 2.2.14.

All treatment concentrations were diluted from stock solutions to working solutions, being 1000 fold more concentrated than intended final concentrations, so that in all treatments 1 µl azakenpaullone per ml seawater was applied (Protocol 2.3.1, step 3). EdU staining of the two control sets confirmed that DMSO, at a concentration of 1 µl/ml for 18 hours treatment had no effect on the amount of proliferating cells, as similar amounts of stained cells could be detected with this method (Figure 5.2.1.1 A and B). To validate visual observations of EdU$^+$ cell numbers, five colonies per treatment were chosen and EdU$^+$ cells counted in comparable sized area. This was followed by statistical analysis, performing two tailed t-tests for each treatment in comparison to SW control.

Statistical analysis of the two control sets displayed no significant difference (p= 0.674219; Table 5.2.1.2), supporting the assumption that DMSO has no effect on proliferating cells at a concentration of 1 µl/ml and a treatment duration of 18 hours. Animals fixed directly after the end of azakenpaullone treatment displayed a slight increase in the amount of stained cells (Figure 5.2.1.1 C) and statistical analysis confirmed the significance of the change (p= 0.049; Table 5.2.1.2). By 24 hours post treatment, a more significant increase of proliferating cells could be observed (Figure 5.2.1.1 D; p= 0.0053; Table 5.2.1.2). By 48 hours post treatment the numbers of proliferating cells decreased again (Figure 5.2.1.1 E) and were statistically indistinguishable from the control (p= 0.11381055; Table 5.2.1.2). These data suggest that the Wnt target gene expression peaks around 24 hours following the end of the azakenpaullone treatment.
Figure 5.2.1.1: EdU staining after azakenpaullone treatment. Scale bar: 200 µm. Numbers of EdU positive cells were unaffected by DMSO, as equal amounts of stained cells were observed in untreated colonies (A) and DMSO controls (B). Following global Wnt activation by azakenpaullone treatment, a slight increase of EdU positive cells could be observed directly post treatment (C), a strong increase of EdU positive cells one day post treatment (D). Two days post azakenpaullone treatment the numbers of proliferating cells appeared to decrease again (E).
Table 5.2.1.2: Statistical comparison of Wnt activated animals with control animals regarding numbers of proliferating cells (EdU\(^+\)) in stolons after azakenpaulione treatments

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<td>0.005358045</td>
<td>0.11381055</td>
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\(^1\): Counted were comparable sections with highest numbers of EdU\(^+\) cells. Shown are counts of EdU\(^+\) cells for five colonies per treatment (n), the average of EdU\(^+\) cells per treatment and the p-value, using a 2 tailed t-test, compared to the seawater control.
5.3.2 Expression pattern after Wnt activation

To investigate which Wnt ligand is a target of Wnt signalling, in situ hybridisation was carried out after azakenpaullone treatment. As Wnt ligands only diffuse short range, special attention was paid on expression in the stolons. The amount of proliferating cells increased significantly 24 hours post treatment and then decreased again (see 5.3.1). Therefore, expression of Wnt target genes should be at their peak around this point in time.

Animals were very fragile after azakenpaullone treatments. This poses a complication for in situ hybridisation, as dirt particles stick to the colonies and need to be vigorously cleaned previous to fixation to avoid dye precipitation during the colourimetric reaction. Proper cleaning was not possible for treated animals, as this led to tissue disruption. In many cases this led to precipitation of NBT/BCIP during the colour reaction, which could mainly be observed at the base of primary polyps, both in antisense and sense probes. However, the unspecific staining was well distinguishable from staining of the Wnt probes. To ensure comparability of unspecific staining, the gentler cleaning regime necessary for treated animals was also applied for seawater and DMSO controls.

Once more it should be emphasised that Wnt ligands are secreted and can affect not only the expressing cell, but also cells in close proximity. Therefore stem cells do not necessarily express the Wnt gene themselves. However, a previous study did show a clear correlation between ectopic Wnt activation and increase of proliferating cells in the stolons (Teo et al. 2006). Hence, Wnt genes expressed in the stolonal system of Hydractinia were categorised as possible candidates of stem cell affecting Wnt genes.

Wnt1

Wnt1 expression was not detectable in the primary polyp or stolon in untreated animals (Figure 5.2.2.1 A) by in situ hybridisation. No up-regulation of expression could be observed after global Wnt activation with azakenpaullone (Figure 5.2.2.1 C-E), suggesting that this gene is not a target of canonical Wnt signalling in stolons. The formation of ectopic tentacles along the body column observed one day post azakenpaullone treatment (Figure 5.2.2.1 D arrow) suggested that global Wnt activation itself was successful. As mentioned in the introduction of 5.2.2, some
NBT/BCIP precipitation occurred during colour reaction due to remaining dirt particles at the base of the primary polyp (Figure 5.2.2.1 star).

Figure 5.2.2.1: *In situ* hybridisation targeting *Wnt1* after azakenpaullone treatment. After azakenpaullone treatment, no expression of this gene could be detected within a 48 hours time course (C/ D/ E). Corresponding sense control is shown in B. The formation of ectopic tentacles along the body column was observed 24 hours post treatment with azakenpaullone (D, arrow). Precipitation at the base of the primary polyp was observed in most colonies (star). Scale bar: 200 µm.
**Wnt2**

Wnt2 expression was already detected at the primary polyp stage previously (see chapter 4.6). To compare expression levels between all different treatments the colourimetric reaction was stopped when one treatment reached strong colouration. This is the reason why SW control did not stain in these experiments as seen previously. Leaving them staining for long enough would result in over-staining of the azakenpaullone treated animals.

Animals fixed directly post azakenpaullone treatment displayed strong expression of Wnt2 (Figure 5.2.2.2 B). Higher magnification revealed that cells within the gastrodermis expressed the gene (Figure 5.2.2.2 D). At this point in time the SW antisense control had just begun to stain (Figure 5.2.2.2 A and C). Animals fixed one day post treatment displayed no expression of Wnt2. These results are not in accordance with the proliferation wave observed one day post azakenpaullone treatment by EdU staining.
Figure 5.2.2.2: Expression of Wnt2 detected by *in situ* hybridisation after azakenpaullone treatment. Strong expression of Wnt2 was observed in animals fixed directly post treatment (B). Higher magnification revealed that expression occurred mainly in the gastrodermis of stolons (D). At this time point the seawater antisense control had just begun to stain in distal parts of the stolon (A). Higher magnification did not elucidate which cells started to express the gene (C). One day post treatment no Wnt2 expression was detectable anymore (E). Slight non-specific NBT/BCIP precipitation was observed at the base of primary polyps (star). Scale bar: A/ B/ E 200 µm, C/ D 50 µm.

**Wnt5B**

Expression of *Wnt5B* was not present in the primary polyp in untreated animals (Figure 5.2.2.3 A). After global Wnt activation with azakenpaullone treatments no expression could be observed after *in situ* hybridisation (Figure 5.2.2.3 C-E), suggesting that this Wnt ligand is not target of canonical Wnt signalling. Unspecific NBT/BCIP precipitation occurred during the colour reaction at the base of primary polyp (star). Ectopic
tentacles were observed in some colonies in azakenpaullone treated colonies (Figure 5.2.2.3 E arrow).

![Figure 5.2.2.3: Wnt5B expression detected by in situ hybridisation after azakenpaullone treatment. No expression of Wnt5B could be observed in untreated animals (A). Also after global Wnt activation no expression of this gene was detectable within a time course of two days (C/ D/ E). Ectopic tentacles were observed in some colonies after treatment (E arrow). Corresponding sense control is shown in B. Scale bar: 200 µm.](image)

**Wnt7**

*Wnt7* expression was not detected in untreated controls (Figure 5.2.2.4 A). This is consistent with previous observations (Chapter4). After azakenpaullone treatments no expression could be observed either (Figure 5.2.2.4 C-E), suggesting that *Wnt7* is not target of canonical Wnt signalling in the stolons. Precipitation occurred during colour
reaction at the base of the primary polyps and in some cases also in the polyp head (star).

Figure 5.2.2.4: Wnt7 expression after global Wnt activation via azakenpaullone treatment. (A) No expression of Wnt7 was observed in seawater antisense control. Also after Wnt activation no expression of Wnt7 could be detected within two days post treatment (C/ D/ E). Corresponding sense control is displayed in B. Precipitation occurred during colour reaction at the base of primary polyps (star). Scale bar: 200 µm.

Wnt8
Expression of Wnt8 was not observed in primary polyps in untreated animals (Figure 5.2.2.5 A). No expression could be observed after global azakenpaullone treatments (Figure 5.2.2.5 C-E), indicating that Wnt8 is no target of canonical Wnt signalling in this context. Precipitation was observed in few colonies during the colour reaction due to remaining dirt particles at the base of primary polyp (star).
Figure 5.2.2.5: Wnt8 expression after global Wnt activation via azakenpaulone treatment. (A) No expression of Wnt8 was observed in seawater antisense control. Also after Wnt activation no expression of Wnt8 could be detected within two days post treatment (C/ D/ E). Corresponding sense control is displayed in B. Precipitation occurred during colour reaction at the base of primary polyps (star). Scale bar: 200 µm.

**Wnt9/10**

In situ hybridisation targeting Wnt9/10 showed no staining in untreated primary polyps or stolons (Figure 5.2.2.6 A). Unspecific NBT/ BCIP precipitation residues were observed at the base of the primary polyp (star) or along the stolon. After global Wnt activation no expression of Wnt9/10 could be detected within 24 hours post treatment (Figure 5.2.2.6 C and D). This lead to the conclusion, that Wnt9/10 is not target of canonical Wnt signalling in the stolons.
Chapter Five – Gene expression after Wnt deregulation

Figure 5.2.2.6: *In situ* hybridisation targeting Wnt9/10 after azakenpaullone treatment. Positive seawater control did not show any expression of Wnt9/10 (A). Precipitation during colour reaction occurred at the base of the primary polyp (star). After global Wnt activation via azakenpaullone treatment no expression of Wnt9/10 was detected within the first 24 hours (C and D). Corresponding sense control is shown in B. Scale bar: 200 µm.

**Wnt11A**

Expression of *Wnt11A* was previously detected at the primary polyp stage (Chapter 4.6). Expression of the gene could be observed in the stolons in untreated seawater control (Figure 5.2.2.7 A). Higher magnification of this area displayed staining of neuron-like cells after *in situ* hybridisation (Figure 5.2.2.7 B), as seen before. Directly post azakenpaullone treatment no change of the expression level could be detected. Many cells in the distal areas of the stolon expressed *Wnt11A* (Figure 5.2.2.7 C and E). Surprisingly, the expression level then decreased dramatically. Only few cells in the stolon tip displayed expression of the gene detected by *in situ* hybridisation one day post treatment (Figure 5.2.2.7 D and F). Also two days post azakenpaullone treatment the amount of *Wnt11A* expressing cells was decreased compared to seawater controls (Figure 5.2.2.7 G and H). This finding suggests that *Wnt11A* is a target of canonical Wnt signalling in *Hydractinia*. 
Figure 5.2.2.7: *In situ* hybridisation after azakenpaullone treatments targeting *Wnt11A*. (A) Untreated animals stained in distal parts of the stolon. Higher magnification revealed that neuron like cells express *Wnt11A* (B). Directly after Wnt activation, expression levels remained similar (C and D). One day post treatment the amount of *Wnt11A* expressing cells decreased dramatically. Only few cells expressing the gene could be detected in the stolon tip (E and F) and remained like this (G and H). Some precipitation could be observed at the base of primary polyps (star). Scale bar: 200 µm.
**Wnt11B**

Unspecific staining was observed in most animals used for *Wnt11B in situ* hybridisation, mainly observed at the base of the primary polyp, where dirt particles stuck to the animals (Figure 5.2.2.8 star). Expression of *Wnt11B* could not be detected in untreated primary polyps (Figure 5.2.2.8 A). After global Wnt activation with azakenpaullone treatment, appearance of *Wnt11B* expression was not detectable within 48 hours post treatment (Figure 5.2.2.8 C/ D and E). The development of ectopic tentacles after treatments showed that Wnt activation itself was successful, therefore it is suggested that *Wnt11B* is not target of canonical Wnt signalling in this context.

![In situ hybridisation targeting Wnt11B after azakenpaullone treatment.](image)

Figure 5.2.2.8: *In situ* hybridisation targeting *Wnt11B* after azakenpaullone treatment. No expression of *Wnt11B* was detected in seawater controls (A), or after treatment within two days (C/ D/ E). Animals two days post treatment exhibited ectopic tentacles (E arrow), indicating that the treatment was successful. Precipitation was observed in most animals at the base of primary polyps (star). Corresponding sense control is shown in B. Scale bar: 200 µm.
Wnt16

Due to the fact that the colourimetric reaction for all different treatments was stopped at the same time to ensure comparability, weaker Wnt16 expression was detected in untreated SW controls in the gastrodermis of primary polyps and stolons (Figure 5.2.2.9 A) than seen previously (Chapter 4.6). Sense control showed no staining, confirming specificity of the probe (Figure 5.2.2.9 B). After azakenpaullone treatment a similar expression level could be observed directly post treatment (Figure 5.2.2.9 C). One day post treatment, at a time when highest increase of proliferating cells are present (Figure 5.2.1.1 D), increase of Wnt16 expression was detected (Figure 5.2.2.9 D). The expression level increased further until two days post treatment (Figure 5.2.2.9 E), and then decreased three days post treatment to below expression level of untreated animals (Figure 5.2.2.9 F). This change of expression level of Wnt16 ligand is mirroring the change of proliferating cells after global Wnt activation (described in chapter 5.3.2).
Figure 5.2.2.9: Wnt16 expression after azakenpaullone treatment detected by in situ hybridisation in *Hydractinia echinata*. Wnt16 is uniformly expressed in the endoderm of the primary polyp and stolons in untreated animals (A). Directly after azakenpaullone treatment similar Wnt16 expression as untreated animals was observed in the stolons (C). One day post treatment increasing expression levels were detected (D), with strongest staining after in situ hybridisation two days post treatment (E). Three days post Wnt activation the expression levels of Wnt16 decreased below expression levels of untreated animals (F). Sense control is shown in B. Staining observed at the tip of tentacle was unspecific, due to sticking dirt particle (E star). Scale bar: 100 µm.
5.4 Wnt inhibition

Global Wnt inhibition was attempted with the compound ZAV939 (Sigma, cat. no. X3004-5MG).

XAV939 inhibits Tankyrase protein (Huang et al. 2009). Tankyrase is responsible for Axin degradation and therefore treatments result in an excess of Axin protein within cells. As Axin levels are no longer restricted through Tankyrase, even in the presence of a Wnt ligand, sufficient amounts of Axin are available within the cell to bind LRP and the “destruction complex”. Therefore the destruction complex is not disrupted during activated Wnt pathway and β-catenin is constantly degraded.

Preliminary experiments were performed on both developing embryos and metamorphosis induced larva to define treatment conditions. Treatments durations were tested for three, five, eight, 10 and 18 hours at final concentration of 1 µM, 2 µM, 10 µM and 20 µM of XAV939 in seawater.

As Wnt signalling is necessary for head development during metamorphosis, tests were also performed to assess the effect of these compounds in metamorphosis induced larva. It was expected that head development would be disrupted after successful Wnt inhibition. Treated animals developed into normal primary polyps or did not survive metamorphosis. This results lead to the conclusion that either the optimal treatment conditions were not found in these attempts, or that the chemical has not the desired effect in Hydractinia echinata.

Embryos treated with XAV939 showed inconsistent effects. For some treatments there was no effect and embryos developed normally, for other treatments there was delayed development or embryos died instantly. Even within same treatments a mixture of all three observed effects could be seen in some cases.
5.5 Summary

It was shown in a past study (Teo et al. 2006), that stem cells react to Wnt signalling in *Hydractinia echinata*, it was attempted in the present study to elucidate which Wnt ligands are themselves target of canonical Wnt signalling and might act as positive or negative feedback loops. Experiments were performed on young colonies of *Hydractinia* and special attention was paid to Wnt ligand expression in the stolonal system, as the majority of stem cells reside in the stolons. It was aimed to identify possible candidates of stem cell affecting Wnt ligands. For this Wnt activation, or inhibition, was performed respectively. The effects on proliferating cells, which are mainly stem cells in the stolonal system, were compared to expression patterns observed by *in situ* hybridisation.

For global Wnt activation a chemical called azakenpaullone was utilised, which was shown in previous studies to up-regulate canonical Wnt signalling by blocking GSK3. The effect of global Wnt activation on the amount of proliferating cells was observed for 48 hours post treatment via EdU staining. EdU incorporated into DNA during S-phase instead of thymidine and can be visualised after fixation of the cells.

Directly post treatment a slight change in the amounts of EdU positive cells could be detected. One day post treatment a clear increase of EdU positive cells was observed. By 48 hours post treatment a slight, non-significant decrease of proliferating cells could be detected, which was below EdU⁺ cells of seawater controls.

To elucidate which Wnt ligand might be displaying a similar expression pattern, and therefore be a Wnt target, *in situ* hybridisation was performed following azakenpaullone treatment. In untreated animals, only *Wnt2*, *Wnt11A* and *Wnt16* expression could be detected at this life stage. Up-regulation of expression after global Wnt activation could be detected for *Wnt2* and *Wnt16*. *Wnt2* expression increased directly post treatments, but could not be detected after 24h post treatment anymore. In contrast, *Wnt16* expression levels mirrored the observed increase and decrease of proliferating cells.

*Wnt11A* expression levels decreased after azakenpaullone treatments. This suggests that this gene acts as a negative feedback loop.
Preliminary experiments of global Wnt inhibition were performed utilising the compounds ZAV939. Collected data was inconclusive, as either the animals developed normally or died. The results permit the following possibilities:

1) The compound tested does not function in *Hydractinia* as expected.
2) The treatment conditions were not suitable and further trials are necessary.
3) Global Wnt inhibition is not compatible with survival of *Hydractinia* during embryonic development and metamorphosis.
6 Chapter Six – Flow cytometry as an analytical method for *Hydractinia* cell biology

6.1 Introduction

Flow cytometry is a method to analyse suspended particles (usually living cells) according to various criteria in a quantitative and qualitative manner.

Use of flow cytometry has increased substantially in medicine and research since its invention. The history of flow cytometry started in the early 1950s when Wallace H. Coulter developed an impedance-based flow cytometry device. This system was later improved by Mack Fulwyler and displays the forerunner of today's flow cytometers (Fulwyler 1965). In 1968/69 the first commercialized cytometers with fluorescence measuring properties were presented from Partec.

Modern flow cytometry can analyse thousands of particles per second. Particles are analysed by laser and grouped according to various parameters such as size, cell granularity (complexity), as well as fluorescent properties. The latter also allows identification of cell surface markers bound to (fluorescent) antibodies (Mandy *et al.* 1995; Orfao *et al.* 1995; Brown & Wittwer 2000). For this method, body liquids, tissues or even whole animals can be used. It is required that all particles are dissociated into a single cell suspension. This suspension can then either be further processed, for example antibody staining and/or other dyes can be added, or be directly loaded into the cytometer. An isotonic sheath fluid creates a laminar flow that facilitates the stream of the suspension. At an interrogation point the particles will pass at least two lasers in a single stream. These lasers intersect passing particles and emitted light passes through filters and dichroic mirrors to various detecting units that can count and measure parameters such as size, cell granularity (complexity), as well as fluorescent properties (Recktenwald 1993). Photomultiplier tubes detect the signals and digitalise them for computer analysis. Therefore FC allows quantitative and qualitative analysis of cell suspensions. Results are most often displayed either as histogram or as a two dimensional dot plot (Mandy *et al.* 1995). Applications are for example blood analysis (Navenot *et al.* 1998; Campbell *et al.* 1999), cell cycle analysis, immunophenotyping (Maslak *et al.* 1994) or cancer assessment (Look *et al.* 1985) in medicine.
Cell suspensions can not only be analysed, it is also possible to separate cell fractions with the help of fluorescent activated cell sorting (FACS). The first cell sorter was constructed in the Herzenberg group at Stanford (Herzenberg et al. 1976). For this, after analysis at the interrogation point, the stream is broken into accurate droplets, each containing a single cell and each droplet can be charged according to chosen parameters. When passing a deflection plate, which creates an electromagnetic field, the added charge of the droplets can direct droplets to different collecting tubes. A schematic of a flow cytometer capable to sort is given in Figure 6.1.1.

Sorting can result in highly pure cell fractions of up to 99% or higher. For example, Figure 6.1.2 shows some components of a blood cell suspension grouped first by size (labelled as FSC) and antibody staining (B220-Cy-Chrome), then by further antibody staining, allowing the different cell types to be indentified and counted. Each dot in the dot plot represents a single cell. Sorting of those different cell types allows further research on specific cell types.
The establishment of flow cytometry would open up a new dimension in *Hydractinia* cell biology, because the entire animal can be dissociated to single cells and analysed by FC. This method could display an invaluable quantitative method to visualise and count cell composition to track cell fate changes after chemical treatments or gene deregulation.

For this study particularly the effect of Wnt signalling on stem cells was of interest. FC would allow quick and easy assessment in a qualitative and quantitative manner to distinguish how stem cells might be affected by Wnt deregulation.
6.2 Experimental approach

To perform flow cytometry (FC) experiments with *Hydractinia echinata* cell suspensions, first an efficient dissociation protocol had to be established. A number of different approaches were attempted. Resulting cell suspensions were evaluated by microscopy and viability staining.

Both alive and fixed cells were examined for the use of flow cytometry. Alive cells rounded up following dissociation and light microscopy allowed identification of all naturally occurring cell types according to their morphology. Also the direct use of cell suspensions following dissociation ensured that no loss of cell population due to washing steps occurred. This means the entirety of cells can be analysed, with the exception of few cells that were damaged during the dissociation process itself.

To produce cell suspension consisting of living cells, a few different methods have been tested. Mechanical dissociation was performed by squeezing polyps through several meshes with different pore size diameters. The resulting suspension was not suitable for flow experiments, as efficiency was rather poor. Many cells had burst during the process and also the presence of cell doublets and even bigger tissue fragments was rather high.

Enzymatic dissociation, which is breaking the bonds between cells, looked more promising. Preliminary dissociation experiments with collagenase and trypsin displayed high dissociation capability, but cells easily burst and cell viability was rather poor. The most efficient dissociation was achieved with Pronase at a final concentration of 0.5 % in seawater (*Protocol 2.4.1*). Resulting cell suspension consisted mainly of single cells, and cell viability assessed by Trypan blue staining was high.

In contrast, when cells were fixed following the dissociation process, it was not possible to exclude the possibility of cell loss during washing steps. Also the fixed cells were reduced in size when transferred into PBS, resulting again in no advantage in comparison to the usage of living cells.

Fixation previous to dissociation was also tested. However, dissociation efficiency was rather low and cells did not round up. This resulted in a lower resolution of cell
populations during FC. The disadvantages specified above made the usage of fixed cells unfeasible for quantitative purpose of flow cytometry. For use during flow cytometry experiments, a few different life stages were utilised. Due to simplicity all optimisation experiments for cytometer parameters were performed with cell suspensions obtained from adult feeding polyps. For this unfed polyps could be cut off colonies and directly be dissociated. Furthermore, adult feeding polyps were utilised for identification of the epithelial cell population. Transgenic Hydractinia (Pln transgenic), which are also expressing green fluorescent protein (GFP) under the control of an epithelial promoter, was available as adult feeding polyps.

However, the main purpose of introducing FC to Hydractinia cell biology was to examine i-cell populations in different context. As mentioned before in this thesis (chapter 1.3 and chapter 4.6), the majority of stem cells reside in the stolons of the animals. Therefore all experiments addressing questions regarding stem cell population were performed on cell suspension obtained from young colonies. In contrast to other life stages, the stolonal tissue can be included during this life stage, as the animals can be grown on glass slides and the amount of chitin within the stolons is still low.

For dissociation either ~ 5 feeding polyps or ~ 12 primary polyps were utilised in 200 µl of Pronase in seawater. The enzymatic reaction was stopped with BSA at a final concentration of 0.1 % in seawater. Cell suspensions were filtered through a 50 µm mesh and concentration calculated using a haemocytometer. Concentration was adjusted to one million cells per ml before assessment by flow cytometry. For all FC experiments, if not otherwise indicated, a BD CantoA cytometer at the National Centre for Biomedical Engineering Science (NCBES) flow facility was used for experiments, using DIVA6 software for digital analysis.
6.3 Flow cytometry of Hydractinia echinata

Following dissociation of *Hydractinia* with Pronase, animals disaggregated into single cell suspensions. Figure 6.3.1 presents an overview of a cell suspension of *Hydractinia*. Several cell types could be identified due to their morphology.

Figure 6.3.1: Light microscopy of *Hydractinia echinata* cell suspension obtained by Pronase digestion. Epithelial cells (star), nematocysts (circle) and stem cells (square) could be identified. Scale bar: 50 µm.

Dilution of the suspension and higher magnification allowed better illustration of several different cell types present in *Hydractinia*. The presence of neurons (Figure 6.3.2 arrow head), epithelial cells (Figure 6.3.2 star), nematocysts (Figure 6.3.2 circle) and stem cells (Figure 6.3.2 square) was observed.
Figure 6.3.2: Higher magnification of *Hydractinia* cell suspension. Epithelial cells (star), neurons (arrow head), nematocysts (circle) and stem cells (square) could be observed. Scale bar: 50 µm.

The enzymatic reaction was stopped and assessed by flow cytometry. All events were displayed in log scale; both forward scatter (FSC) and side scatter (SSC). FSC axis corresponds to the size of the cells and SSC to their complexity (granularity).

After initial experiments, starting parameters for the cytometer were chosen as following:
FSC: 8 Volt
SSC: 320 Volt
FITC: 500
FSC area scale: 0.55
Threshold: 1200

Compensation for performance variations of the cytometer was conducted with size beads for each experiment as mentioned in protocol 2.4.2 (Figure 6.3.3). All events representing particles smaller than the 3.4 µm bead were excluded from analysis as
this is supposed to represent cell debris and background noise. All events above the FSC log scale of $10^4$ were considered to represent cells. For beads 20 000 events were recorded, and 100 000 events were recorded for cell suspensions.

![Figure 6.3.3: Flow cytometry of utilised size beads displayed as dot plot and histogram. (A) Contour dot plot, displaying the three different size beads (3.4 μm/ 7.4 μm/ 14.7 μm) are shown. (B) Histogram of beads highlights that the peak of the 7.4 μm bead is well aligned with the log scale of $10^4$. All events representing particles above a size of 3.4 μm are counted for digital analysis of occurring events (P1).](image)

After adjustment of cytometer parameters with size beads *Hydractinia* cell suspensions were analysed under the same conditions. Several experiments using young colonies or cut feeding polyps were performed to confirm reproducibility of the dissociation protocol and cytometer parameters.

All particles recorded, that were bigger than 3.4 μm, were included in the analysis of cell suspensions (Figure 6.3.3 Fraction P1, Figure 6.3.4 red colour). Young colonies displayed normally two distinct groups of cells in dot plot results (Figure 6.3.4 B and D). Tight gates were drawn manually around these cell groups. P2 (Green colour) displays the potential group of epithelial cells. Comparison with bead size position suggests that the diameter of cells within this gate is supposed to be around 15 μm. This is in accordance with epithelial cells identified by light microscopy (Figure 6.3.1 and 6.3.2, star). The complexity is high, entailed to high granularity of these cells. The second distinct group, P3, which is displayed in blue, was hypothesised to contain stem cells. These cells feature a diameter of six to ten μm, with low complexity and a big nucleus. Events illustrated in pink (P4) do not display cells, as size of around 3 μm already displays background noise, consisting of cell debris and non-cellular particles.
Figure 6.3.4: Flow cytometry of two independent *Hydractinia echinata* cell suspensions from young colonies, plotted using the parameters of FSC (corresponding to size) and SSC (corresponding to complexity). Displayed are normal dot plot and contour dot plot for each experiment respectively (A and B; C and D). All events representing particles bigger than 3.4 μm are displayed in red (P1). The hypothesised epithelial cell fraction is displayed in green (P2), suggested stem cell fraction in blue (P3). Particles with a diameter around 3.4 μm are displayed in pink (P4) and are supposed to be cell debris or small non-cellular particles.

Quantitative analysis of events was conducted in hierarchy diagrams of populations (Figure 6.3.5). The two data sets of young colonies are shown as examples out of several experiments performed, indicating similar percentage rates for distinguished cell groups. Therefore results of dissociation and FC procedure are assumed to be reproducible in young colonies.
Figure 6.3.5: Corresponding hierarchy diagrams of the two flow cytometry data sets shown in Figure 6.3.4. The two distinguished cell groups exhibit similar percentage rates (Boxes), indicating reproducibility of performed dissociation and FC procedure.

Experiments were also performed with cut feeding polyps. Results of two different sets of data are shown (out of numerous experiments performed) as dot plot diagrams in Figure 6.3.6 and hierarchy diagrams in Figure 6.3.7. Identical gating strategy to the young colony suspensions was utilised to permit comparability between the suspensions for the two different life stages.

Similar to experiments conducted with young colonies, the suggested stem cell fractions of feeding polyps (P3) display comparable percentage rates to each other (Figure 6.3.7 A and B, black box). However, the used gate of potential epithelial cells (P2) is not optimal for feeding polyps. Percentage rates for P2 in hierarchy diagrams display high differences (Figure 6.3.7 A and B, red box). Close examination of the contour dot plots (Figure 6.3.6 B and D, black box) illustrate that this fractions exhibit a much higher spread of complexity and therefore accurate comparison between these fractions is not captured by the utilised gating strategy.
Chapter six – Flow cytometry as an analytical method for Hydractinia cell biology

Figure 6.3.6: Flow cytometry of two independent Hydractinia echinata cell suspensions from cut polyps plotted using the parameters of size (FSC) and complexity (SSC). Displayed are normal dot plot and contour dot plot for each experiment respectively (A and B; C and D). All events representing particles bigger than 3.4 μm are displayed in red (P1). The hypothesised epithelial cell fraction is displayed in green (P2), suggested stem cell fraction in blue (P3). Particles with a diameter around 3.4 μm are displayed in pink (P4) and are supposed to be cell debris or small dirt particles. Black boxes in (B) and (D) highlight higher complexity spread that is not captured with used gating strategy.

Figure 6.3.7: Corresponding hierarchy diagrams of the two flow cytometry data sets shown in Figure 6.3.6. The suggested stem cell groups (P3) exhibit similar percentage rates to each other (Black box). The fractions of suggested epithelial cells (P2) of the two data sets display high degree of difference (Red box). Therefore applied gating strategy is not to be considered effective to capture all epithelial cells.
One working hypothesis for stem cells in *Hydractinia echinata* is that pluripotent stem cells reside mainly in the stolonal system and become progressively more determined to a specific cellular fate as they migrate into the polyp.

A comparison between suggested stem cell fractions in FC, purely analysed by parameters of size and complexity, displayed higher percentage rates of cells within P3 in young colonies (~21.9 %) containing stolonal material, compared to feeding polyps (~ 18.7 %). This finding supports the hypothesis that more stem cells can be observed in stolons.

Further investigation of suggested cell fractions was needed. To confirm the identity of epithelial cell fraction, FACS of *Pln*-transgenic animals was performed. These animals ectopically express *Pln* (An *Oct4* homologue) as well as a GFP fusion-protein under an epithelial-specific promoter (Millane *et al.* 2011). Cell sorting by FACS was also performed from cell suspensions of young colonies for both, suggested epithelial cells and stem cells. Sorted fractions were then examined with light microscopy.
6.4 FACS of wild type and *Pln* transgenic animals

FACS experiments were performed by Dr. Shirley Hanley of REMEDI using a BD FACS Aria II cell sorter. For this the entire system was set up with artificial seawater to prevent a phosphate buffered saline (PBS)-mediated osmotic shock to the *Hydractinia* cells (osmolarity of seawater is 3x higher than standard PBS) and preserve them for further analysis by light microscopy. Applied cytometer settings were adjusted for the different cytometer type to:

- Sheath: artificial seawater
- Threshold: 5,000
- Flow rate 1.0: 2,000 events/sec
- 0-32-0 sort mask
- Agitation: 300 rpm
- FSC/SSC: 137/255; 1.5NDF; 100 μm

The cell suspension was analysed and sorting gates were then manually drawn around the two distinct cell groups. Potential epithelial cells were named as “big” and suggested stem cell fraction was named “medium” (Figure 6.4.1).
Figure 6.4.1: Contour plot and chosen gates for FACS of young colony cell suspension. Analysis was performed with the parameters of size (FSC) and complexity (SSC). Sorting gates were manually drawn around two distinct cell groups. Proposed epithelial cell fraction was named “big”, suggested stem cell fraction was named “medium”.

Sorted cell fractions were then evaluated by light microscopy. Sorted fractions for proposed epithelial cells were very pure. Nearly all cells examined displayed a diameter of 15 to 20 μm and contained high numbers of granule (Figure 6.4.2 A). The morphology of these cells highly supports the hypothesis of this cell fraction being epithelial cells, because identification of cell types in Hydractinia is mainly based on morphology. Clear identification of many cell types in Hydractinia, based on gene expression and antibody staining, is difficult. Examination of the putative stem cell fraction contained mainly cells with a diameter around 7 μm and contained very few granule and a large nucleus (Figure 6.4.2 B). This would support the hypothesis that this fraction contains stem cells.
Figure 6.4.2: Light microscopy of sorted cell fractions. (A) Cells found in the potential epithelial cell fraction (“big”) contained almost exclusively cells with the morphology of this cell type. (B) Examination of the suggested stem cell fraction was comprised mainly of cells with the morphology of stem cells. Scale bar: 50 μm.

However, purity was not as good as it was for the epithelial cell fraction. Cells that clearly were no stem cells could also be observed in this cell fraction (Figure 6.4.3). Lower numbers of neurons, nematoblast and small cells containing high numbers of granule could be found.
Figure 6.4.3: Light microscopy of sorted “medium” cell fraction also contained, in addition to cells that resemble stem cells, other cell types that clearly belonged to other cell lineages. Few neurons, nematoblasts and small cells containing higher numbers of granule were observed. Scale bar: 50 μm.

To further support the identification of epithelial cell fraction, FACS and FC were performed using Pln transgenic feeding polyps. Pln transgenic animals were generated by Dr. Cathriona Millane in our group previously (Millane et al. 2011). These animals express the Pln gene under the control of an epithelial promoter fused with GFP. As utilised transgenic animals are mosaics, most but not all epithelial cells possess fluorescent properties that can be detected in the FITC channel of cytometers when excited by blue laser. Figure 6.4.4 displays such a transgenic feeding polyp excited by blue light.
FC analysis of dissociated *Pln* feeding polyps displayed similar, but not identical, group patterns in comparison to wild type animals, regarding the parameters of size and complexity. Differences detected are most likely due to the expression of the transgene, supporting the hypothesis that FC should be capable to detect cell composition changes after gene deregulation. Observed were the two cell groups seen previously in wild type analysis, plus one additional group close to the suggested epithelial cell fraction. The suggested epithelial cell fraction was highlighted within a red box (Figure 6.4.5 A). The same data set was then analysed with the parameters of size and green fluorescence (Figure 6.4.5 B). Cells suggested to be epithelial cells displayed strong positive signal of fluorescence (Red box).
Chapter six – Flow cytometry as an analytical method for Hydractinia cell biology

Figure 6.4.5: Flow cytometry of transgenic Pln feeding polyps. (A) Contour dot plot using the parameters of size (FSC) and complexity (SSC). Potential epithelial cell fraction is marked within red box. (B) When replacing the parameter of complexity against green fluorescence (FITC), suggested epithelial cell fraction displays strong positive signal for green fluorescence.

To assess this cell group further, FACS was performed by Dr Hanley of REMEDI. Applied sorting strategy was to sort out cells with a diameter around 15 μm and strong fluorescent signal (Figure 6.4.6 A, GFP**).

Figure 6.4.6: FACS of Pln transgenic animal cell suspension. (A) Dot plot of the suspension was analysed by parameters of size (FSC) and green fluorescence (Alexa Fluor 488A). Sorting strategy was to separate big cells with strong fluorescent signal (GFP**). (B) Histogram illustrates that nearly all detected GFP signals are included in sorted cell fraction.

The obtained cells were then examined under a fluorescence microscope. These cells indeed comprised a diameter between 15 and 20 μm and a strong green fluorescent signal (Figure 6.4.7 A and B). Based on morphology, the cells were positively identified as epithelial cells. Uptake of blue dye during viability check by trypan blue staining suggested that cells were no longer viable.
Figure 6.4.7: Light and fluorescent microscopy of GFP++ cells of *Hydractinia Pln* transgenic animals, sorted by FACS. Morphology of obtained cells in GFP++ fraction confirms suggested identity as epithelial cells (A). When excited by blue light, strong detection of GFP could be observed (B). Scale bar: 50 μm.

Results of light microscopy confirm suggested epithelial cell fraction in FC experiments.
6.5 Flow cytometry after Wnt activation

A first attempt to track down cell fate changes by FC was conducted using *Hydractinia echinata* young colonies. To investigate if the increase of cell proliferation observed in the stolonal system after Wnt activation (Chapter 5.2) is due to the presence of more stem cells, flow cytometry was performed using azakenpaullone treated colonies. The aim was to elucidate whether these cells are stem cells and would result in a measurable difference of suggested stem cell fraction in a quantitative manner by FC. To ensure that observed effects are due to Wnt activation, DMSO controls were set up for each treatment examined, in addition to untreated seawater control. Azakenpaullone treatments were performed as described in protocol 2.4.1 step one to seven. Independently each treatment was repeated twice and consisted of around 12 replicates. Colonies were then dissociated according to the protocol described in 2.5.1 and the resulting cell suspensions analysed with cytometer settings mentioned in protocol 2.5.2 and cytometer adjustments stated in chapter 6.3. To verify that treatments were successful, additional treatments were stained with EdU (Complete protocol 2.4.1, followed by EdU staining, protocol 2.3.2; Data not shown).

Obtained cell suspensions were examined by light microscopy. As no cells smaller than a diameter of 7 \( \mu \text{m} \) were observed, FC gating was chosen to exclude all events representing particles smaller than the 7.4 \( \mu \text{m} \) size bead from the analysis with DIVA6 software (Gate P2) (Figure 6.4.1).

![Figure 6.5.1](image)

Figure 6.5.1: Flow cytometry of utilised size beads displayed as dot plot and histogram. (A) Contour dot blot, displaying the three different size beads (3.4 \( \mu \text{m} \), 7.4 \( \mu \text{m} \), 14.7 \( \mu \text{m} \)) are shown. (B) Histogram of beads highlights that the peak of the 7.4 \( \mu \text{m} \) bead is aligned with the log scale of 10\(^4\). All events below the 7.4 \( \mu \text{m} \) size bead were excluded from digital analysis of occurring events (P2).
Dot plots of seawater control (Figure 6.5.2) and the different treatment (Figure 6.5.3) cell suspensions all displayed two distinct cell groups.

Figure 6.5.2: Contour dot plot of *Hydractinia* cell suspension, seawater control. Two distinct cell groups can be identified.

Figure 6.5.3: Contour dot plots of *Hydractinia* cell suspensions, DMSO controls (upper row) or azakenpaullone treatments (lower row). All cell suspensions displayed two distinct cell groups. Gates were manually drawn around epithelial cell fraction (P4) and suggested stem cell fraction (P3).

For all cell suspensions percentage rates of the two cell populations (Figure 6.5.4) were then examined. For both, suggested stem cell fraction (P3) and epithelial cell fraction (P4), percentage rates were not stable within DMSO controls. Therefore reasonable conclusions could not be drawn, even though previous experiments showed clearly that DMSO had no effect on cells at utilised concentration and
treatment duration. The control group used for EdU staining showed no visible differences (Data not shown; For EdU data see chapter 5.3).

Table 6.5.1: Table of percentage rates for gated cell fractions P3 and P4 in relation to all events measured within gate P2. Experiment consists of two independent experiments and 12 replicates each.

<table>
<thead>
<tr>
<th></th>
<th>SW stem cells</th>
<th>SW epithelial cells</th>
<th>DMSO stem cells</th>
<th>azaken stem cells</th>
<th>DMSO epithelial cells</th>
<th>azaken epithelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>direct</td>
<td>48.5%</td>
<td>17.4%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d post</td>
<td>55.2%</td>
<td>47.9%</td>
<td>15.4%</td>
<td>17.1%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2d post</td>
<td>43.3%</td>
<td>46.3%</td>
<td>19.4%</td>
<td>18.7%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3d post</td>
<td>43.8%</td>
<td>43.4%</td>
<td>20.1%</td>
<td>21.2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50.5%</td>
<td>52.9%</td>
<td>17.8%</td>
<td>16.2%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

As controls showed significant variation and azakenpaullone treatments did not reflect effects seen with EdU staining, a conclusive statement regarding cell fate changes can not be made at this point in time. Experiments to use this method for quantitative analysis for cell compound changes after gene deregulation and chemical treatment were not yet successful.
6.6 Summary

Flow cytometry is an interesting and useful tool to assess cell suspensions quantitatively and qualitatively. To enable this method for *Hydractinia* cell biology, a dissociation protocol was generated and optimised. Preliminary experiments with *Hydractinia* cell suspensions were promising. However, to this date, only two distinct cell groups could be identified. Stable and reproducible results could be observed for suspensions generated from either young colonies or feeding polyps. To secure stable results for epithelial cell fraction, a less tight gating strategy is inevitable than used in this study. With the help of fluorescence activated cell sorting (FACS), it was possible to confirm the suggested epithelial cell fraction. Both wild type and *Pln* transgenic animals were utilised for this. Particularly the transgenic animals were useful, as transgenic epithelial cells fluoresce green under blue light excitation. Sorted cell fractions were subsequently examined by light microscopy. Morphology of cells included in potential epithelial cell fractions resembles characteristics of epithelial cells. In addition, suspensions generated from *Pln* transgenic animals, sorted by positive fluorescence and size contained exclusively epithelial cells. This strongly supports the positive identification of epithelial cell fractions in FC experiments. Sorted cell fractions for epithelial cells were highly pure. In contrast to this, examination of the suggested stem cell fraction revealed, that in addition to stem cells, other cell types were sometimes detected within this cell fraction. This suggests that further refinement of cytometer parameters might be necessary. To do so, extensive trials with FACS, both in log and linear scale need to be conducted. Current experiments did not produce conclusive data to utilise this method for tracking of cell fate change.
Chapter seven - Discussion

7 Discussion

In my project I have studied Wnt signalling in the cnidarian *Hydractinia echinata*. In *Hydractinia* only two Wnt ligands had been described previous to this study, *Wnt3* and *Wnt5A* (Plickert *et al.* 2006; Stumpf *et al.* 2010). In other cnidarians, such as *Hydra* and *Nematostella*, putative members of nearly all Wnt subfamilies have been reported. Therefore, it was to expect that *Hydractinia echinata* should also possess additional Wnt ligands.

Wnt signalling seems to be well conserved throughout the Metazoa and has important roles in development and adult tissue homeostasis (Gordon & Nusse 2006). However, the evolution of this important signalling pathway is yet unclear (Logan & Nusse 2004). It was suggested that all currently present Wnt subfamilies have evolved prior to the splitting event of Bilateria and Cnidaria, as members of all Wnt subfamilies but Wnt9 have been identified in *Nematostella* (Kusserow *et al.* 2005; Guder *et al.* 2006). Additionally, members of many Wnt subfamilies are present in *Hydra* (Lengfeld *et al.* 2009; Philipp *et al.* 2009). The hypothesis of formation of the Wnt subfamilies in the last common ancestor of Bilateria and Cnidaria however, was based on very little data of cnidarian representatives. Therefore further analysis including a bigger dataset was needed to corroborate or reject this assumption. Studying Wnt signalling in *Hydractinia echinata* presented me with the opportunity to obtain further data that might help to address this question.

Another aspect of interest comprises the question of the ancestral functions of Wnt signalling. Wnt is reported to have important roles in diverse functions, such as organogenesis, patterning of the nervous system, stem cell decision-making and formation of the body axis. It was already implied in the literature that the developmental mechanisms of establishment of the body axis is an evolutionary inheritance from a shared ancestor of bilaterian and cnidarian organisms (Primus & Freeman 2004; Kusserow *et al.* 2005; Guder *et al.* 2006; Lengfeld *et al.* 2009). Data generated from *Hydractinia* also support the hypothesis of ancient function of Wnt in body patterning (Plickert *et al.* 2006; Duffy *et al.* 2010). Using *Hydractinia* as a study subject allowed not only to investigate the expression patterns of the newly identified Wnt ligands in the whole animal throughout the life cycle, but might also deliver further clues to the evolution of this pathway and its ancestral functions.
This study also aimed at identifying possible candidates for a stem cell controlling Wnt ligand. Involvement of Wnt signalling in stem cell decision-making was shown in many different contexts and organisms (Nusse 2008). Many multipotent stem cells were described to be controlled by Wnt signalling, such as haematopoietic stem cells (Reya et al. 2003), intestinal stem cells (Pinto & Clevers 2005) or skin stem cells (Huelsken et al. 2001). Furthermore, pluripotent stem cells are supposed to be controlled by Wnt signals as well (Sato et al. 2004; Reya & Clevers 2005). A stem cell affecting function of Wnt in other cnidarian representatives has not been reported yet. A previous study conducted in *Hydractinia* revealed that stem cells express Frizzled, the Wnt receptor (Teo et al. 2006), and therefore are able to react to Wnt signalling. However, the two so far described Wnt ligands are unlikely candidates for stem cell controlling Wnt ligands, as they are not expressed in the stolons, where the majority of the stem cells reside. Identification of involved Wnt ligands, followed by functional studies would give further information if a controlling function of Wnt on stem cells is ancestral and predates the split of Cnidaria and Bilateria.

Additionally, in the literature contradictory studies can be found whether or not Wnt signalling is maintaining stem cell self renewal or leads to differentiation. While Sato et al. argue that Wnt expression is sufficient to promote self-renewal (2004), Dravid et al. report that Wnt alone is not sufficient (2005). Furthermore, other studies suggest that Wnt does not promote stem cell self-renewal, but actually leads to stem cell differentiation (Kielman et al. 2002; Muroyama et al. 2004). Therefore investigating Wnt signal functions in a basal invertebrate might help in resolving the contradiction.

Finally, it was attempted in this study to establish flow cytometry for *Hydractinia* cell biology, a method that counts and analyses particles such as cells. This method could provide a useful tool to assess the cell composition of the whole animal. Comparisons between wild type animals and gene deregulated animals should provide further clues regarding functions of Wnt signals in cell fate decision-making.
7.1 Identification of new Wnt ligands

Nine novel Wnt ligands in *Hydractinia echinata* that are expressed throughout its life cycle have been identified with the help of an EST and draft genome databases. In addition to the previously described *Wnt3* and *Wnt5A* genes, putative homologous for the Wnt genes *Wnt1*, -2, -5B, -7, -8, -9/10, -11A, -11B and -16 were identified. The majority of the coding sequences of all the genes were obtained with the help of RACE PCR. The gene fragments were purified by gel electrophoresis and verified by sequencing. Gene 5’ or 3’ termini that could not be amplified have been added with the help of the genome and transcriptome databases that became available to me only at the end of this study. BLAST searches were performed for all different genes, resulting in hits for respective Wnt subfamilies. This suggested that identified genes indeed belong to the Wnt super-family. Additionally, it was interesting to examine whether or not *Hydractinia* Wnt genes could possibly be homologous to Wnt genes from other cnidarians and bilaterians. Putative members of all Wnt subfamilies described in *Hydra* could also be identified in *Hydractinia* (Table 7.1.1). However, my findings also indicate that the Wnt subfamilies of cnidarians do not correspond clearly to the Wnt subfamilies described in bilaterians.

<table>
<thead>
<tr>
<th>Wnt protein</th>
<th><em>Hydractinia echinata</em></th>
<th><em>Hydra</em> spp</th>
<th><em>Nematostella vectensis</em></th>
<th><em>Platynereis dumerilii</em></th>
<th><em>Drosophila melanogaster</em></th>
<th>human</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Wnt1</em> / wingless</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td><em>Wnt2</em> (13)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>2X</td>
</tr>
<tr>
<td><em>Wnt3</em></td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>2X</td>
</tr>
<tr>
<td><em>Wnt4</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>Wnt5</em></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>2X</td>
</tr>
<tr>
<td><em>Wnt6</em></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>Wnt7</em></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>2X</td>
</tr>
<tr>
<td><em>Wnt8</em></td>
<td>X</td>
<td>X</td>
<td>2X</td>
<td>X</td>
<td>X</td>
<td>2X</td>
</tr>
<tr>
<td><em>Wnt9</em> (14/15)</td>
<td></td>
<td>3X</td>
<td></td>
<td></td>
<td>X</td>
<td>2X</td>
</tr>
<tr>
<td><em>Wnt9</em> / 10</td>
<td></td>
<td>X</td>
<td>3X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Wnt10</em></td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>2X</td>
</tr>
<tr>
<td><em>Wnt11</em></td>
<td>X</td>
<td>2X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td><em>Wnt16</em></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td><em>WntA</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>
Chapter seven - Discussion

It was suggested by other researchers that all major Wnt subfamilies had already formed previous to the emergence of Cnidaria and Bilateria (Kusserow et al. 2005; Guder et al. 2006). To further examine relationship and homology of *Hydractinia* genes, gene alignment and phylogenetic analysis was performed. Data obtained from *Hydractinia* and other representatives of the Hydrozoa, first time presented in this study, do not support the common hypothesis of joint evolution of Wnt subfamilies.

When the different *Hydractinia* Wnt genes were aligned with Wnt genes previously annotated as members of the same subfamily in different invertebrate and vertebrate representatives, high degrees of sequence similarity with hydrozoan genes was observed. This suggested close relationship of these genes. Phylogenetic analysis of hydrozoan Wnt genes resulted in well supported clusters, suggesting that the majority of present hydrozoan Wnt subfamilies evolved previous to the divergence of the different orders of Hydrozoa. Phylogeny allowed, within the Hydrozoa, a confident establishment of orthologous relationship. In contrast to hydrozoan Wnt genes, alignment with other cnidarian classes and vertebrate species featured very low degrees of sequence similarity. In accordance with this, phylogenetic comparison with representatives of other metazoan species, including representatives of anthozoan cnidarians, casted doubt on the scenario of an evolution of Wnt subfamilies previous to splitting of Bilateria and Cnidaria. Low node values in the phylogenetic tree were often observed between hydrozoan gene clusters, anthozoan, scyphozoan and bilaterian genes. In other cases the phylogenetic analysis positioned hydrozoan gene clusters far away from putative bilaterian and anthozoan counterparts (Subfamilies Wnt7 and Wnt16). Taken together, phylogenetic analysis in many cases failed to recover statistical evidence for orthologous relationships of Wnt subfamilies between Hydrozoa, representatives of other Cnidaria classes and Bilateria. It is conspicuous that all examined hydrozoan species feature a loss of the subfamilies Wnt4, Wnt6 and WntA. Furthermore, all hydrozoans included in analysis contained Wnt genes that could not clearly be assigned to either the Wnt9 or Wnt10 subfamily. In contrast, in *Nematostella* putative members of the subfamilies Wnt4, Wnt6 and WntA are identified. Additionally, a member of the Wnt10 subfamily is described. However, this protein did not cluster with the hydrozoan Wnt9/10 proteins.
Chapter seven - Discussion

Even though putative members of nearly all described bilaterian Wnt subfamilies have been suggested to exist within the phylum Cnidaria (Table 7.1.1), I rather propose a lineage specific evolution of Wnt subfamilies, including extensive loss of gene families, gene duplication and sequence divergence within cnidarian evolution, as already the comparison to anthozoan Wnt proteins did not support orthologous relationship.
Chapter seven - Discussion

7.2 Gene expression patterns

Expression patterns of the different Wnt ligands are only discussed in comparison to expression patterns found in other cnidarian species, as these animals exhibit similar developmental stages and body plans. Furthermore, phylogenetic analysis of the Hydractinia Wnt genes did not show close relationship to their homologues in other metazoan species. Even though phylogenetic analysis did also not support homology of Wnt genes between hydrozoans and representatives of other cnidarian classes, conservation of function is possible due to similar body plans.

Nearly all newly identified Wnt genes in Hydractinia are expressed ubiquitously in the embryo, in contrast to the previously identified Wnt3 transcript in Hydractinia, which is asymmetrically expressed on one side of the embryo only. Of these Wnt ligands Wnt1, -5B, -7, -9/10 and Wnt16 were expressed until gastrulation, while Wnt8 and Wnt11B were only weakly expressed after fertilisation of the egg and were not detectable after few cell divisions. This suggests that these two Wnt transcripts have a function only during early development, while the other expressed Wnt genes have a role throughout embryonic development. Wnt2 mRNA had a higher concentration around the nucleus and this expression pattern was maintained for several cell divisions.

The early expression of Wnt genes in Hydractinia is in contrast to Wnt expression in Nematostella, as in this animal Wnt mRNAs are not detectable until gastrulation (Kusserow et al. 2005). In Hydra nothing is known about embryonic Wnt expression pattern, as the embryo develops in a cyst and all attempts of in situ hybridisation have failed (Fröbius et al. 2003).

In Nematostella, Wnt genes were observed to be expressed at the planula larva staggered along the anterior/posterior axis, similar to Hox-cluster observed in Bilateria (Kusserow et al. 2005). In Hydractinia this could not be observed. At the larva stage Wnt1 was expressed in few cells at the posterior side of the animal, similar to the previously identified Wnt3. Wnt5B and Wnt16 were expressed in the anterior side of the larvae, but excluded from the posterior side, while Wnt7 and Wnt9/10 remain ubiquitously expressed throughout the animal.
Chapter seven - Discussion

After metamorphosis induction, Wnt1 and Wnt2 were expressed in the posterior tip of the larvae in *Hydractinia*, similar to the two already described Wnt genes (Wnt3 and Wnt5A). This could mean that Wnt1 and Wnt2 have a similar function as Wnt3 throughout metamorphosis. Only Wnt7 remains broadly expressed throughout the larval stage and after metamorphosis induction. However, a slight increase of expression level could be observed in the most posterior located gastrodermal cells. The function of Wnt7 at this life stage remains to be uncovered.

After completion of metamorphosis, expression in the stolons was examined. As the majority of stems cells are thought to reside in the stolonal system in *Hydractinia* (Müller et al. 2004; Teo et al. 2006) and Wnt signalling functions often over short range (Moon et al. 2004), expression of a stem cell affecting Wnt ligand should be detectable in close proximity of stem cells. Of all identified Wnt genes only Wnt2, -11A and -16 expressions could be observed in the stolon. Wnt2 expression was detected in distal parts of the stolons, where either a new polyp-bud was about to form, or the stolon was about to branch out. Therefore a role of this Wnt gene in proliferation processes can be hypothesised, as proliferation control by Wnt signalling was described in literature (Nusse 2008). However, no functional studies have been conducted and further investigation is necessary. Wnt11A expression was observed in distal parts of the stolons in cells that appeared morphologically to be neurons, as the stained cells exhibit neurite-like extensions. This was the only site where this gene was expressed. Therefore, a function in stolon specific neurons could be assumed. Wnt16 expression was observed in the entire gastrodermis of stolons and the primary polyp. The function of Wnt16 remains unsolved, but the fact that expression could be observed in the whole stolonal system makes this gene a good candidate to affect stem cells, which mainly reside in the epidermis of stolons. As expression was detected in the gastrodermis, the proteins would need to diffuse through the mesogloea to affect stem cells in the epidermis. However, as Wnt’s are known to act as diffusible morphogens this poses a possible scenario. No comparison to *Nematostella* and *Hydra* is possible for stolon specific Wnt expression, as these cnidarian species are not colonial and therefore do not form stolons. Moreover, no Wnt data on any other colonial cnidarian regarding expression in stolons has been published so far.
Chapter seven - Discussion

In adult feeding polyps the expression of the newly identified Wnt1, -2, -7, -9/10, -11B and -16 were detected in *Hydractinia*. Wnt1 expression was observed in the oral tip of the head, similar to *Hydractinia Wnt3*. This expression pattern is also in accordance with expression of the Wnt1 transcript in *Hydra* and *Nematostella* (Kusserow *et al.* 2005; Lengfeld *et al.* 2009). It can be hypothesised that this gene has an ancient function in head specification in cnidarians and that this is the ancestral function of Wnt1 in this phylum.

In contrast, Wnt2 expression was observed in a belt-like pattern in the body column within the epidermis. This expression pattern is in accordance with Wnt2 expression in *Nematostella*, where the transcripts also can be found in the epidermis below the oral pole (Kusserow *et al.* 2005). In *Hydra* Wnt2 is expressed only in early formation of polyp buds (Lengfeld *et al.* 2009). Because both *Nematostella* and *Hydractinia* share the same Wnt2 expression pattern, it is likely that the different Wnt2 expression pattern in *Hydra* is a derived character.

*Hydractinia* Wnt7 could be detected in the broad head area, which is a similar expression pattern to *Hydra* Wnt7 (Lengfeld *et al.* 2009), but in contrast to *Nematostella* Wnt7 expression, which is restricted to the oral pole (Kusserow *et al.* 2005). Therefore it can be assumed that broad head expression of this Wnt gene might be hydrozoan specific.

The neuronal expression observed for *Hydractinia* Wnt9/10 seems, so far, to be unique in this animal. In *Nematostella* no members of the Wnt9 subfamily have been reported (Kusserow *et al.* 2005). A Wnt10 gene has been described in *Nematostella*, which is expressed in individual cells in the endoderm (Kusserow *et al.* 2005). The dissertation of Kusserow (Kusserow 2005) locates these cells in mesenteries and developing pharynx. In *Hydra* Wnt9/10a and Wnt9/10c expression seems to be restricted to few cells in the hypostome (Lengfeld *et al.* 2009). Three different Wnt9/10 ligands have been reported in *Hydra*. However, which cells express these genes has not been indicated.

Wnt11B transcripts were observed at high levels in the oral tip and broadly expressed in lower degrees throughout the epidermis of the feeding polyp. The function of this
gene remains unsolved. In *Nematostella* and *Hydra* only one *Wnt11* gene is known, respectively. Expression of these genes was reported in tentacle endoderm (*Nematostella*—(Lee et al. 2006)) or in the oral tip (*Hydra*—(Lengfeld et al. 2009)) in adult feeding polyps. These expression patterns are in contrast to the broad expression found in *Hydractinia*. However, in both, *Nematostella* and *Hydra*, only one *Wnt11* gene was reported. Therefore the second identified *Wnt11* gene in *Hydractinia* could be a novelty in the Hydractiniidae lineage.

*Hydractinia* *Wnt16* was detected in adult feeding polyps in a belt-like pattern in the epidermis of the body-column. This expression pattern is in contrast to expression observed of *Wnt16* homologues in *Hydra* and *Nematostella*. In these animals *Wnt16* is expressed in the head, either in the oral tip or in ectoderm of the pharynx, respectively (Kusserow et al. 2005; Lengfeld et al. 2009).

In gonophores two *Wnt* genes were expressed, *Wnt2* and *Wnt16*. Interestingly, they were restricted to males. While *Wnt2* expression could only be observed in very young gonads, the *Wnt16* gene was also weakly expressed in more mature ones. This expression pattern is consistent with a role of Wnt signalling in spermatogenesis in *Hydractinia*. In *Hydra*, forming gonads express high levels of the Wnt inhibitor *GSK3* (Rentzsch et al. 2005; Nishimiya-Fujisawa & Kobayashi 2012). Therefore one can assume that Wnt signalling is generally down-regulated during gametogenesis in *Hydra*. I have not studied *GSK3* expression in *Hydractinia* and therefore can only speculate about possible Wnt down regulation in oogenesis.

Overall, Wnt expression patterns in *Hydractinia* are much more diverse than in *Hydra*. While Wnt expression is mainly observed in the oral side of the *Hydra* polyp, suggesting functions in head determination and axial patterning (Lengfeld et al. 2009), Wnt expression in *Hydractinia* could be observed not only in the head, but also in the body column and stolonal system. Additionally, in *Hydractinia* Wnt expression could be described in neuron-like cells (*Wnt9* and *Wnt11A*). Influential function of Wnt signalling on neuronal tissue was shown in vertebrates in many studies (McMahon & Bradley 1990; Muroyama et al. 2004; Caricasole et al. 2005; Salinas & Zou 2008). The fact that neuron-like cells in *Hydractinia* express Wnt genes could imply that neuronal functions of Wnt developed early in the evolution of Wnt pathway. In *Nematostella*, also
ectodermal Wnt expression has been suggested to have a strong bias to neuro-ectodermal expression domains (Kusserow et al. 2005), supporting the idea of ancient functions of Wnt in neuro-ectodermal tissues. Finally, links between Wnt signalling and stem cell control have been shown in numerous studies (Alonso & Fuchs 2003; Clevers 2006; Aoi et al. 2008; Katoh 2008; Nusse 2008). The presence of frizzled receptor in stem cells of *Hydractinia* also suggest a controlling function of Wnt signals in a stem cell context in this basal organism (Teo et al. 2006). The Wnt ligand working upstream has not been identified yet.

However, the functions of Wnt expression in *Hydractinia* proposed in this study are all based on morphology and positioning of expressing tissues and are therefore mere speculations. Gene expression dedicated to cell lineages has not been established in many cases. Extensive investigations by functional studies are demanded to establish justified hypothesis regarding functions of different Wnt ligands in *Hydractinia*. 
7.3 Expression patterns after Wnt deregulation

It was shown in a past study that stem cells in *Hydractinia* express *Frizzled*, the Wnt receptor, and are therefore thought to be competent to respond to Wnt signalling (Teo *et al.* 2006). This finding is in accordance with observations in other animals, where it was reported that Wnt signalling controls either stem cell self-renewal (Sato *et al.* 2004; Reya & Clevers 2005) or leads to cell differentiation (Kléber & Sommer 2004; Muroyama *et al.* 2004).

However, the two Wnt genes that have been described so far in *Hydractinia* are unlikely candidates for this aspect of Wnt function, as they are not expressed in the stolons, where the majority of stem cells reside. Three genes, *Wnt2, -11A and -16* were expressed in stolons. To gain further insight into this issue I applied a commercial GSK3 inhibitor, which activates Wnt downstream events and studied the expression of *Wnt* genes after the treatment. The rationale of these experiments was that *Wnt* genes that activate the pathway in a specific context might also be its target, forming either a positive or negative feedback loop. *Wnt3*, for example, is known target of itself in *Hydra* and in *Hydractinia* head context (Broun *et al.* 2005; Plickert *et al.* 2006; Duffy *et al.* 2010; Nakamura *et al.* 2011). It has to be stressed that modulation of a *Wnt* gene expression is not necessarily a proof for its involvement in pathway activation.

After global Wnt activation, using the GSK3 inhibitor azakenpaullone, a clear increase of proliferating cells could be observed one day post treatment. The amount of proliferating cells decreased two days post treatment. This confirms a previous study (Teo *et al.* 2006). *In situ* hybridisation experiments revealed, not surprisingly, that *Wnt2, -11A and -16* changed their expression in stolons following Wnt activation. None of the other identified Wnt genes displayed expression in the stolons following global Wnt activation, suggesting that they are unlikely to affect stem cells in stolonal context. Both, *Wnt2* and *Wnt16* displayed an increase of expression level after treatment of azakenpaullone, while *Wnt11A* displayed a decrease of expressing cells one day post azakenpaullone treatments. *Wnt2* expression was increased directly post treatments and dropped to below detection levels one day post treatment - at a time when a clear increase of proliferating cells was detectable with EdU staining. In contrast, *Wnt16* expression level mirrored the cycle of increase and decrease of proliferating cells. It is possible that *Wnt2, -11A and Wnt16* jointly activate canonical signalling in stolons, but
that $Wnt11A$ forms a negative feedback loop, while $Wnt2$ and -16 form positive loops. The data presented in this study was generated by global Wnt activation. Separate gene deregulation experiments are needed to propose more substantiated suggestions regarding stem cell effects of particular Wnt genes. As Wnt genes are described to have pivotal roles during embryonic development, the establishment of inducible over-expression vectors would be necessary to examine Wnt effects in all life stages. This includes extensive work of microinjection to generate transgenic animals that have the over-expression constructs stably introduced into their genome. Another possible approach would be treatments with ectopic Wnt protein and examine effects. Additionally, further work on cell identity within uncompromised tissues is needed. So far cell identity is mainly based on morphologic properties of cells. Gene expression based identification of cells would allow more precise examination; however, this is not yet established for many cell types. For example, RFamides or LWamides are only expressed in fractions of the neuronal cells of Hydractinia (Plickert et al. 2003). A pan-neuronal gene expression has not been identified so far. A statement made in Plickert et. al. (2003) is still valid: “Considering non-availability of genetic analysis and the so-far limited success in expressing transgenes in hydroids, gene functions are difficult to analyse in hydroids”.

Experiments aiming to globally inhibit Wnt signalling were attempted. While a Wnt inhibitory effect of XAV939 was shown in other organisms (Fearon 2009; Huang et al. 2009; Liu & He 2010), data from preliminary experiments in Hydractinia, using either developing embryos or metamorphosing larva, was inconclusive. This suggested that either the treatment conditions, duration or concentration, were insufficient or the compound was not working as expected in Hydractinia. As it was shown in different species that Wnt signalling has pivotal roles during development (Logan & Nusse 2004), it is possible that successful global Wnt inhibition is lethal. However, preliminary experiments conducted in Hydractinia did not result in death of treated animals in all cases. Instead, effects on animals appeared to be random. All possible outcomes - no effect, delayed development or death - were observed in some treatments within the same approach. These finding rather suggest insufficient treatment conditions or difficulties with solubility of the compound in seawater. Therefore more experiments are needed targeting specific Wnt ligands at life stages of interest. This could be carried
Chapter seven - Discussion

out by RNAi treatments, which have been successfully performed for other genes in _Hydractinia_ previously (Duffy _et al._ 2010; Millane _et al._ 2011).
7.4 Flow cytometry as a new analytical tool for *Hydractinia* cell biology

Flow cytometry (FC) is widely used in mammalian cell biology to analyse cell suspensions in a quantitative and qualitative manner, but has hardly been used in marine invertebrates for this purpose so far.

To utilise FC technique, I optimised a dissociation protocol for *Hydractinia echinata*. The resulting cell suspension contained all known *Hydractinia* cell types. Preliminary flow cytometry experiments for young colonies, however, displayed only two distinct cell groups. These groups could be observed in multiple experiments and were reproducible regarding their position within dotplots and their relative proportions of the total cell numbers. The reproducibility of cell groups within adult feeding polyps was less satisfactory. While the amount of stem cells was comparable between different experiments, the group of epithelial cells showed high degrees of difference between experiments. These findings are plausible if it is taken into consideration that both, gastrodermis and epidermis are formed by epithelia, but exhibit very different functions. While the epidermis poses protection to outer environment, the gastrodermis is responsible for food particle uptake and therefore is likely to contain higher percentages of granule than epidermis cells. As primary polyps used for flow experiments were fed only once and then starved two days prior to dissociation, the amount of granule contained in gastrodermal epithelial cells is likely to be less high than in gastrodermal cells of adult feeding polyps, as feeding polyps were starved only 24 hours prior to dissociation.

The remaining cell types could not be resolved under utilised FC conditions and were probably dispersed within the two main cellular groups.

Based on the parameters of size and complexity I hypothesised that one group was primarily composed of epithelial cells (Size around 15 µm and high complexity) and the other group to contain stem cells (Size around 6-8 µm and low complexity). However, the stem cell population probably contained committed cells and other cell types.

The parameters of size and complexity turned out to be insufficient to gain higher resolution of contained cell groups. As transgenic lines, expressing genes and fluorescent reporter systems under the control of cell type specific promoters, are not
yet available for *Hydractinia echinata*, resolution could not be increased utilising fluorescent properties. Also antibody staining coupled to fluorescent dyes could not be applied to living cell analysis, due to the fact that the cells have to remain in seawater (osmotic pressure) and antibody staining requires PBS based environment. A boost of resolution should be possible by applying linear scaling to the FSC and SSC parameters. During trials with linear scaling higher numbers of cell groups could be observed. Though, reproducibility was not given and therefore further extensive experiments are needed to establish linear scaling for FC utilising *Hydractinia* cell suspensions.

The hypothesis of cell group identity was also tested by fluorescence activated cell sorting (FACS) of wild type and *Pln* transgenic animals (Millane *et al.* 2011), followed by light microscopy examination. This confirmed the cell fraction of epithelial cells. Purity of the cell fraction was very high. The second cell fraction observed in FC analysis was hypothesised to contain stem cells, according to small size and low complexity. Light microscopy of sorted cell fraction confirmed that many cells within this fraction were indeed i-cells, but, as noted above, also contained other cell types. This suggests that the flow parameters need further optimisation. For this, extensive trials with both, log and linear scale are required to ensure better fine-tuning. As only the parameter of size and complexity (Cell granularity) can be utilised at the moment for all cell types with the exception of epithelial cells, improvement of cytometer parameters is difficult. It would be helpful to use transgenic lines that express fluorescent proteins in different cell lineages, which were not available for my study, except the *Pln* animals that expressed a reporter GFP in their epithelial cells.

I attempted to use FC to track down cell composition changes after Wnt deregulation. However, the results were inconclusive. This is most certainly due to the lower resolution of the second cell fraction, which contains the stem cells. Cell fate changes that might occur after global Wnt activation are possibly not detectable at the moment as these changes might affect cell types that are contained in this group.
Chapter seven - Discussion

7.5 Concluding remarks

The presented study addresses Wnt signalling in the basal cnidarian *Hydractinia echinata*. Little is known about the evolution of this important pathway. Therefore more data of phylogenetically basal animals is needed to provide further insight into the origin of Wnt signalling.

During this study I was able to identify nine novel Wnt genes in *Hydractinia*. Phylogenetic analysis of these genes was carried out by Dr. Paulyn Cartwright (University of Kansas) and suggested rather lineage-specific ramification of Wnt genes. This is in contrast to all published work in recent years that favour a pre-bilaterian ramification. No statistical evidence for pre-bilaterian evolution of Wnt subfamilies could be observed in new datasets including more cnidarian data. Therefore I propose that a more careful approach is needed when comparing putative gene homologues. To shed further light into the evolution of Wnt subfamilies more data is necessary from basal invertebrates.

The present study suggests that while Wnt ligand subfamilies evolved separately in different lineages, the overall functions of Wnt signalling seem to be conserved throughout the animal kingdom. The previously reported two Wnt genes in *Hydractinia* play a role in axial polarity, as reported for Wnt genes in other metazoan animals. A function in stem cell decision-making was suggested previously (Teo *et al.* 2006). However, a Wnt candidate for this task has not been identified. I propose that the newly identified Wnt2, -11, -16 are possible candidates to fulfil this role in *Hydractinia*, as they are expressed in close proximity to stolonal stem cells. Expression in neural lineages seems to be ancestral for Wnt genes as well, as Wnt9/10 and Wnt11A expression was observed in neurons in the head or stolons, in *Hydractinia* respectively. Wnt expression in neurons was reported in several species throughout the animal kingdom.

Functional studies are necessary to test the here suggested involvements of Wnt in different functions such as stem cell decision-making.
8 References


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Kirikoshi H., Sekihara H. & Katoh M. (2001b) WNT10A and WNT6, Clustered in Human Chromosome 2q35 Region with Head-to-Tail Manner, Are Strongly Coexpressed in SW480 Cells. *Biochemical and Biophysical Research Communications* 283, 798-805.


Symmons O. & Spitz F. (2013) From remote enhancers to gene regulation: charting the genome’s regulatory landscapes. Philosophical Transactions of the Royal Society B: Biological Sciences 368.


References


9 Appendix

9.1 Solution recipes

**Lysis buffer:**

500 μl Solution D  
500 μl Phenol pH 4  
100 μl 2 M Na-acetate  
7.7 μl beta-mercaptoethanol

**Solution D:**

To 19.53 ml of ddH₂O add  
16.66 g Guanidinium thiocinate (final concentration 4 M)  
1.17 ml 0.75 M Sodium citrate (final concentration 25 mM)  
1.76 ml Sarcosyl (N-lauroyl sarcosine, final concentration 0.5 %)

**Fixing solution**

For 50 ml  
1.1955 g Hepes 0.1 M Hepes pH7.5  
0.0246 g MgSO₄ 2 mM MgSO₄  
1.227 g NaCl 0.42 M NaCl

**1 x PBS** (Phosphate-buffered Saline):

For 1 L:  
8 g NaCl 137 mM NaCl  
0.2 g KCl 2.7 mM KCl  
1.44 g Na₂HPO 10 mM Na₂HPO  
0.24 g KH₂PO₄ 2 mM KH₂PO₄

**1 x PBST:**

For 50 ml:  
50 ml PBS (1X PBS pH 7.5)  
50 μl Tween 20
Appendix

Hybridisation Mix:

For 10ml:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 ml Deionised Formamide</td>
<td>50 %</td>
</tr>
<tr>
<td>2.5 ml 20 x SSC</td>
<td>5 x SSC</td>
</tr>
<tr>
<td>1 ml 1 mg/ml Heparin stock</td>
<td>0.1mg/ml</td>
</tr>
<tr>
<td>1 ml 1 mg/ml tRNA stock solution</td>
<td>100µg/ml</td>
</tr>
<tr>
<td>10 µl Tween 20</td>
<td>0.1 %</td>
</tr>
<tr>
<td>0.5 ml DEPC H₂O</td>
<td></td>
</tr>
</tbody>
</table>

20 x SSC:

For 1 L:

<table>
<thead>
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</thead>
<tbody>
<tr>
<td>175.3 g NaCl</td>
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</tr>
<tr>
<td>88.2 g Sodium Citrate</td>
<td></td>
</tr>
</tbody>
</table>

AP-buffer:

For 10 ml AP-buffer:

<table>
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<th>Concentration</th>
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</thead>
<tbody>
<tr>
<td>1 ml 1M NaCl</td>
<td></td>
</tr>
<tr>
<td>1 ml 1M Tris-HCl                      pH 9.5</td>
<td></td>
</tr>
<tr>
<td>1 ml 500 mM MgCl₂</td>
<td></td>
</tr>
<tr>
<td>7 ml DEPC water</td>
<td></td>
</tr>
<tr>
<td>10 µl Tween 20</td>
<td></td>
</tr>
</tbody>
</table>

Sterile filter to avoid precipitate.

In situ hybridisation staining solution:

In 1 ml of AP buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5 µl 50mg/ml NBT in 70% dimethylformamide</td>
<td></td>
</tr>
<tr>
<td>3.5 µl 50mg/ml BCIP in 100% dimethylformamide</td>
<td></td>
</tr>
</tbody>
</table>

Lavdovsky’s fixative:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 ml formaldehyde</td>
<td></td>
</tr>
<tr>
<td>2 ml acetic acid</td>
<td></td>
</tr>
<tr>
<td>25 ml ethanol (100 %)</td>
<td></td>
</tr>
<tr>
<td>20 ml water</td>
<td></td>
</tr>
</tbody>
</table>

----------------------

50 ml
Sörensen’s buffer:

0.2 M pH 7.4.

Solution 1: Use 35.61 g Na₂HPO₄·2H₂O and make up to 1000 ml with distilled water, stir until dissolved.

Solution 2: Use 27.6 g NaH₂PO₄·H₂O and make up to 1000 ml with distilled water, stir until dissolved.

Add 40.5 ml of solution 1 to 9.5 ml of solution 2 to give 50 ml 0.2 M phosphate buffer and test with pH-meter that the pH is at 7.4.

10X MOPS electrophoresis buffer:

- solve 41.8 g MOPS in 700 ml DEPC water
- Adjust to pH 7 using 2 M NaOH
- Add 20 ml of 1 M sodium acetate (in DEPC water)
- Add 20 ml of 500 mM EDTA (pH 8; in DEPC water)
Bring to a final volume of 1L with DEPC water
9.2 Gene data

9.2.1 Phylogenetic analysis

*Hydractinia* Wnt genes clustered well with their putative orthologues of other hydrozoans species in well-supported clades, suggesting that the identification and affiliation into the different Wnt subfamilies was coherent within the Hydrozoa. However, relationships with Wnt’s outside the Hydrozoa could not be established. As a matter of fact, none of the cnidarian Wnt’s could be assigned to any bilaterian Wnt subfamily with high statistical support, suggesting that the naming of cnidarian Wnt’s is rather arbitrary.
Figure 9.2.1.1: Phylogenetic tree of hydrozoan Wnt protein including only hydrozoan datasets. *Hydractinia* sequences are underlined. Nodes $>70$ are considered well-supported. Nodes below 70 are considered unsupported in analysis. Hv: *Hydra vulgaris*, Hm: *Hydra magnipapillata*, Pc: *Podocoryne carneae*, Ch: *Clytia hemisphaerica*, El: *Ectopleura larynx*, Ph: *Polypodium hydriforme*, Hs: *Hydractinia symbiolongicarpus*, He: *Hydractinia echinata*
9.2.2 Wnt1

Figure 9.2.2.1: Sequencing results of *Hydractinia* Wnt1.
Figure 9.2.2.2: Full coding sequence of *Hydractinia* Wnt1 ligand.

Figure 9.2.2.3: Amino acid sequence of Wnt1.

Figure 9.2.2.4: Alignment of *Hydractinia echinata* Wnt1 protein and *Hydra vulgaris* Wnt1 protein. 

"*" (asterisk) indicates positions which have a single, fully conserved residue. 
"." (colons) indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.

"." (dots) indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix.
Figure 9.2.2.5: Alignment of *Hydractinia echinata* Wnt1 protein and different *Hydra* Wnt proteins. "*" (asterisk) indicates positions which have a single, fully conserved residue (highlighted in green). ":" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. "." (dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix. Amino acid residues occurring in four out of five sequences are highlighted in orange.
9.2.3 Wnt2

Figure 9.2.3.1: Sequencing results of *Hydractinia* Wnt2.
Appendix

Figure 9.2.3.2: Coding sequence of *Hydractinia Wnt2*.

```
ATGAAAGCATTACCAGTGGAAATTCCTCTCTTGTTATCCTCAACGTTATATCCATAATATCAGTGGTGGCTTCAAAGCTTAAGTTCACAAGTAATATCCATTGGGTCGATGGGCAGTTGCAACAACATAGCAGGTTTAGTGAAAGAACAGAAAGTATTATGCAGAAAATATCCAACACTAATGAAAGTTGTAGCAAATGGTGCACACCTTGGTTTATCAGAATGTCAACATCAGTTCTCTACTTCAAAATGGAATTGTTCTCATGTTGATCCAAGACAGAAAGGACTGCTCGGTCTATTGACCAGTCAAGCCAATAGGGAAGCTGCTTTTTTCCAAAGCAATAACATCAGCTGGAGTTGTAGTTAAGGTCACTCAAGCATGCAATTCTGACAAATACCATCGAGCCTGCACTTGCGACTACAAAAATGCTGGAAGGAAAGGGAAAGATTTTCAATGGAACAGCTGCAACGATAATGTCAATTTTGGCATGACGTTTGCGCAGAAATTTCTTGATGCACGTGAAATTGGAAACGATGCA CGGGTGCTCAATGAACAAAACAAACAAACGTACGCGGCAAGACTGCGTCTGCTGTAAGAAAGAAACAGTCGAAATGTGAGCTGTCGTTCTGGCTCATGCAATGTCAAGACATGTCGCTACACCTTGTCAGAGTTTATCGCGTCGGACACCATCTGCAATAAGCCTATGTAGAGGCGAGTTGCTGTCGAGTTGCTGTTAGACCAAAAAC
```

Figure 9.2.3.3: Amino acid sequence for *Wnt2* gene.

```
MKAFSTSGNSSLVFLTLIHQVTSSWLLQSSLSSQVISIGSMSCNIAGLVKEQVKLCRKYPLEMKVMVANGAHLGISEQHOFSTSCKWNCHVDPQKGLLGILTSQANREAAFFQAIATSAGVVKVTVQACNSDKYHRACT CDYNAGRKGKDQWNSCDNVKFGMFTFQFLDAEIGNARVLMNKQNLAGRLAVKLECTC HGVGSQSNVTCTRYLSEFYVRGQDLHNYSEAAAVMLQTQNNLVPKSIAEKFRKRNVLFELESPDPCY CVKDLKEGTLGAVRRCNKTPGTGCEIMCCGMFGHTKKVWVESNCNSFIWCCVKTQCTCQEVQKH CFRSIIKRRKFRNKHDEPREKNLVSITEEGKLIQRDPQOKLNVDLDNMYKKT
```
Figure 9.2.3.4: Alignment of *Hydactinia echinata* Wnt2 protein and *Clytia hemisphaerica* WntX1A protein. 

"*" (asterisk) indicates positions which have a single, fully conserved residue. 

":" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.

"." (dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix.
Figure 9.2.3.5: Alignment of *Hydractinia echinata* Wnt1 protein and different *Hydra* Wnt proteins. "*" (asterisk) indicates positions which have a single, fully conserved residue (highlighted in green). ":" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. "." (dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix. Amino acid residues occurring in four out of five sequences are highlighted in orange.
Figure 9.2.4.1: Sequencing results of *Hydractinia* Wnt5B.

Figure 9.2.4.2: Left agarose gel picture of PCR product. Right lane full coding sequence of *Hydractinia Wnt5B.*
Figure 9.2.4.3: Amino acid sequence of Hydractinia Wnt5B.

![Amino acid sequence of Hydractinia Wnt5B](image)

Figure 9.2.4.4: Alignment of Hydractinia Wnt5B protein and Xenopus laevis Wnt5B protein.

‘*’ (asterisk) indicates positions which have a single, fully conserved residue. ‘:’ (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. ‘.’ (dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix.
Figure 9.2.4.5: Alignment of *Hydractinia echinata* Wnt5B protein and different *Hydra* Wnt proteins (highlighted in green). "*" (asterisk) indicates positions which have a single, fully conserved residue. ":" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. "." (dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix. Amino acid residues occurring in four out of five sequences are highlighted in orange.
Figure 9.2.4.6: Alignment of published Hydractinia echinata Wnt5A protein and newly identified Hydractinia Wnt5B protein. "*" (asterisk) indicates positions which have a single, fully conserved residue. ":" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. "." (dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix.
Figure 9.2.5.1: Sequencing results of *Hydractinia* Wnt7 fragment.
Figure 9.2.5.2: Left lane agarose gel picture of PCR product, right lane displays the coding sequence of *Hydractinia Wnt7*.

Figure 9.2.5.3: Amino acid sequence for *Wnt7*.  

```
ATGAATCTTTTCGTACTTATTATTTTTATCAAATATTATGTGGTTTTAATTCTATTGTAAGAATCTTTAGTAAACCTGCTGGAGAAATCAGAGGAGTGGTTGCTATCAGAGGAGGGAAGTTACACAGTGTAACAGGAGCTTTGGGAGAATGAGATGGAACTGTACAAACCAATCGACTGGTAAAATAAATGTTCTATTTGGGAATCGTCATGGCAAGAGGTTCAAGAGAAGCCGCTGTCATGTTCGCCATGGCATCAGCCGGTATGTTGAAAGAATCGCATCTGAGTCATACCGAGAACGCCGCAGAAAAAAGAAATCAAACTGTAAGGGGTTGTTACCAACTACACGGCGCTCGAAACGCGCGACCTTTTGTCAGCACTAATGATTGTGACACCGACATTGATATAGGTGATACATATGCTCGGAAAGTCATGGAAAATTGGCTAACGACACAGAAATCAAGTAAGGAGAATCTCATGAATATACATAATGCTCGTGTTGGACGATTAGCAGTGCGACAGCATATGAAGAAAGTTTGTAAATGCAACGGTTTATCAGGAGCGTGCAATGCCCTATGTTGCTATAAAGATTTACGACCGCTTAGTGTAAGTACTGATGGTTGAAGAAACAATACAAGAATGCGAAAAAGGTAAAAACAGGACCACTGGTAAAACGAGATGGATTTAGATTACTGGTAACTGCAAAAGATTCTATA
```

Figure 9.2.5.4: Amino acid sequence for *Hydractinia Wnt7*.  

```
MNLFVIIKIMCFNSIETSIRKLENLATEVCÖKHQMKKLGYNLQRPFLNLCQESNTLFTEAINGFKQGİTQC
RSFGRMRWNCTQSTGKINVLFHVSMARGSSRRAAVMFAMASAGMVERIASESYRERRRKKNQTVRGC
YQLHGARNARAFVSTDCTDIDGTYARKVMEWLTTQKSSNEENLMHNRVGLAVRQHMKKC
KCNGLSGACNALCYYKDLRPSVSTAWLKKYKNAKVKTVTPRLKRÐGFDRLVTDISIAPEADJYLYH
DSPNYCLHDPRTGSLTTGRQRLNNTDNDNCSMCGRGYETHVMARĐEQÇKSTRFÇCWCVESTQTC
RRLYTITHRCK
```

Figure 9.2.5.5: Amino acid sequence for *Wnt7*.  

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234
Figure 9.2.5.4: Alignment of *Hydractinia echinata* Wnt7 protein and *Hydra vulgaris* Wnt7 protein.

"**" (asterisk) indicates positions which have a single, fully conserved residue. "": (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.

"." (dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix.
Figure 9.2.4.6: Alignment of *Hydractinia echinata* Wnt7 protein and different *Hydra* Wnt proteins (highlighted in green). "*" (asterisk) indicates positions which have a single, fully conserved residue. ":" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. "." (dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix. Amino acid residues occurring in four out of five sequences are highlighted in orange.
9.2.6 Wnt8

Figure 9.2.6.1: Sequencing results of Hydractinia Wnt8
Figure 9.2.6.2: Left lane agarose gel picture of PCR product. Right lane coding sequence of *Hydractinia Wnt8*.

Figure 9.2.6.3: Amino acid sequence for *Wnt8* gene.
Figure 9.2.6.4: Alignment of *Hydractinia echinata* Wnt8 protein and *Hydra vulgaris* Wnt8 protein.

"*" (asterisk) indicates positions which have a single, fully conserved residue. ":" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.

"." (dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix.
Figure 9.2.6.5: Alignment of *Hydractinia echinata* Wnt8 protein and different *Hydra* Wnt proteins. "*" (asterisk) indicates positions which have a single, fully conserved residue (highlighted in green). "::" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. "." (dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix. Amino acid residues occurring in four out of five sequences are highlighted in orange.
Figure 9.2.7.1: Sequencing results of *Hydractinia* Wnt9/10 fragment
Figure 9.2.7.2: Left lane agarose gel picture of PCR product. Right lane coding sequence of *Hydractinia Wnt9/10* ligand.

Figure 9.2.7.3: Amino acid sequence for *Wnt9/10* gene.
Figure 9.2.7.4: Alignment of *Hydractinia echinata* Wnt9/10 protein and *Hydra vulgaris* Wnt9/10b protein.

"\*\*\*" (asterisk) indicates positions which have a single, fully conserved residue. "\*\*" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.

"\.*" (dot): indicates conservation between groups of weakly similar properties - scoring ≤ 0.5 in the Gonnet PAM 250 matrix.
Figure 9.2.7.5: Alignment of *Hydractinia echinata* Wnt9/10 protein and different *Hydra* Wnt proteins. **""** (asterisk) indicates positions which have a single, fully conserved residue (highlighted in green). **":"** (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. **."** (dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix. Amino acid residues occurring in four out of five sequences are highlighted in orange.
Figure 9.2.8.1: Sequencing results of *Hydractinia Wnt11A*.
Appendix

ATGGCACAATACTATACATAATTCTTTCTTATCATGGCATGTTGCATGCATGTGTTTGCTATAAAATGGCTGAACGAGCCACTTAGAGATGTGAAAAAATGGTCTCGACCAGCCTGTACATCAGAGAACTTTCATTTGAACGCAAACAGCGCGGCATATGCCGCCACCAGACAAATTACATGGATTCCGTTTCCAGTGGCGTGAGATACAATATCGAGTTGCAGAACTTTATTTCAAGAGGACTCTTGGAATTGCTACAACATTCTGAAGCTCCACATTTTGATTACAATATAAATAATGCAACCAAAGAATCAGCTTTCGTGCACGCCCTGTCTGCTGCCGCTCTTGCATATCACGTGGCCACCGACTGTGCGTCTGGAAAGATAGCAAACTGTAGATGTTCCACCCGGTATCAAGGAGGGGTAACAACGCTGCCGAATGTTCCTGGTAAAGTGATATTATACAGTAGAGCGTGTCCTCACGTTTATGAGAAAGGAATTCACTTTGCCAGAAAGTTCCTTTCACCCGGAGAGAAGAAATTCAAAAAGGTGGCTAAAAAATCGCCTTACCGCGCTGGCCAGTTGAAAATTGCAGAACATAACAAGAGGTCGGATATAAGGTTTTTCGTACTGAAAAATACAAATACATAAAATGTGGTTGCAGAGGTAATACTGGATCGTGTCCAATCAAAACATGTTATCAGTCCATTGATTATTTCCCCGTTATGGCAAAAGAAATACGCAAAAGATATGAAGAGGCATCTAGAGTAGCGATCGACAAAAAAACAGGTGCGTTGACTCCAACCAATGGGATTGATGAAATCGTACCAGAACAAGAACTGATCTACTCCGATAATTCTTTAAAGAAATGCTCAACCAAAAAAGCATCGAACAAGACATGCGCTATAGAAGGTGTAGTTTGGATCCAAACGCAAAAGACTACTGTAAAAAGATGTGCTGTGATGGAAAACACAAAGCAACGGAGATTGTTGTGGCAGTCCAATGCAGTTTAATTCGTCTACTGCTGTCGTGTCGATTGTCAATGGTGCAATCATAAGAAAATGGTGCAAATCTGATT

Figure 9.2.8.2: Confirmed full coding sequence of *Hydractinia Wnt11A* ligand.

MAQYYTKFFLIMACCMMHVFAIKWLNEPLRDVKKWSRPACTSENHFNLNAKQRIGCRHTQNYMDSDVSSGAA DTISSCRTLFQEDSWCYNLKAPHFDYNINNATKEAFHAVSHAALAYHAVDATDASIGKIANCRSTRYQ GGVTLPNVPKVLYSRACPHVYEKGHFARKFLSPGEKKFKVAKKKRPGQLIAEHNREVGYKVFRTEKYKIKCCCGTNGTSCPIKTCYSSIDYFPVMAKEIRKRYEASVRAIDKXTALTNPINDIEVPQEQLI YSDNSLKKSTKSKARTDMYRRCSDLPNAKDYCKKMCDDGKHKATIIVAVQCSCKFVYCCRDQW CNHKMVQICI

Figure 9.2.8.3: Amino acid sequence for *Wnt11A* gene.
Appendix

Figure 9.2.8.4: Alignment of Hydractinia echinata Wnt11A protein and Hydra vulgaris Wnt11 protein.

"*" (asterisk) indicates positions which have a single, fully conserved residue. ":" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.

"." (dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix.
Figure 9.2.8.5: Alignment of *Hydractinia echinata* 11A protein and different *Hydra* Wnt proteins. "*" (asterisk) indicates positions which have a single, fully conserved residue (highlighted in green). ":" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. ":" (dot): indicates conservation between groups of weakly similar properties - scoring =< 0.5 in the Gonnet PAM 250 matrix. Amino acid residues occurring in four out of five sequences are highlighted in orange.
9.2.9 Wnt11B

Figure 9.2.9.1: Sequencing results of *Hydractinia* Wnt11B fragments.
**Appendix**

### Figure 9.2.9.2: Coding sequence of *Hydractinia* Wnt11B fragment.

```
ATGACCAGATTAGGATTGTGGATAATTGCAATCGAATGTTGGAATGATCAAAACATTATGGTT
TTGGGCTTACAAATACGGAATATTGTCGTCGATACACGCGTTTGAATTTGGCAGAAGGTATGGA
TTTCACTGGAAAAGGAAGGCTGCCAGGAGAAGATGTTGGATGAGATATAGGGACATATCTGA
AGAAGGCGCAGTTTATTTGAGCAGAAACTTCCGCGTTTGCGCTCAAACTTCACTGTTTT
CCGACGCTTCTGACAAGATGTTTCTCCGAGGAGGAATATGTCGTCGACGGTTAATTTGGA
TTGGCGTTACAGAAATACTCCAATAACGAAATTTGGGCGGTACAGCAAGTGTGTACAAAAGATTATGG
TTTCACTGGAAAGCAAGTTCGCTTCTGCAAAAACACATTTTCCATGGATGACATTTGTACAGGAGGCTG
TAATTGACGCTAAGGAGGAGTGCATACACCATTTGCACAAACAAAAGTGGGATTGTCACGTGATAAA
AGAAGCGCCAACATTTAATGAGGATCTGAAAAAGGACACAATACAATCCGCGCTTGTTTATGCTCTAT
CGGCAGCCTCATTGGCTATCACAGTATCACGACGATGCAAAATGGGGCAAATTCCAGATTGTTCATG
TCAACACAACAGGAAGATCTTTTCTCCATCAGATCTTGTTATAATATACAAATT
GGACTAAGAGGGCTTGGTACTGTTGGAAGAATGAACTTTGGGTATGACTGAGACACGGTGCTGTTG
CCGAAATATTTGTTTATAGAAGGTTATATACCTGAGTTAAGGCGGCAGGGTGTTAATTTGGAAGAAGGT
TCAAAAGAACGCGCTAATTTTCTCCGATGACTCTTACCTGCTCCTCAGAGGGCTCACTAATAAGGTTT
AGATGTCGAAAATTACCTATCAGATAAAGATGGAACACGTGGGAAAGGGTGTACATTTGCTGAGAATACA
GACCCAGAGTGTCGCTACACTGGACGACGTTGCTGCTCTGATATACTAAAAACGAGATTGTTGAGA
ACTTGTTGTGGTGAATGTTGGAATATGTCGTCGACGGTTAATTTGGAAGAAGGT
```

### Figure 9.2.9.3: Full amino acid sequence for *Hydractinia* Wnt11B protein.

```
MTRLWMILIIAIYIPNVKCIWLQKYSNNEIWANVQCTDKYGTGKQVRFCFTHFPWTMTFVQEAVID
AKEECHIPHLHHKQWDNYKEIAKAPFTNEKLDDIQVYALSASLAIVTSRCKMGQIPDCSCHNRV
SPFSSIVNSLSDNKKICPKCGIVQNGKFAQTIIFNLGFLPKNERQAEEEIAVRKHIQIGKLGTEEV
CVTGATGGGCPRKFKYRRIIPNEAANVIFERYKVKVLFHPDNRPPKASLNKEMSKLPQYIKDEHVDK
LYYLEDTQQCATTGRRCVLDISKPDSCENLCCGRGKYRGLIEVNSCRCRFVYCCVKVCDNCISMFKQV
FECL
```

### Figure 9.2.9.4: Alignment of *Hydractinia echinata* Wnt11B protein and *Hydra vulgaris* Wnt11 protein.

```
```

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**250**
Figure 9.2.9.5: Alignment of *Hydractinia echinata* Wnt11A and Wnt11B protein. “*” (asterisk) indicates positions which have a single, fully conserved residue. “:” (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. “.” (dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix.
Figure 9.2.9.6: Alignment of *Hydractinia echinata* 11B protein and different *Hydra* Wnt proteins. "*" (asterisk) indicates positions which have a single, fully conserved residue (highlighted in green). ":" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. "." (dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix. Amino acid residues occurring in four out of five sequences are highlighted in orange.
Figure 9.2.10.1: Sequencing results of Hydractinia Wnt16.
### Appendix

**Figure 9.2.10.2:** Full coding sequence of *Hydractinia Wnt16* ligand.

```
ATGCAAAATCTTGCTGCTTGGGAGTTTTATATTGTTTTTCTTACCAACAGATTCGCTGAGTCAGCAGTG
GATGAAACTAGCGTTGGTATCTGGTGATATATACAACAGAAAGTTTTGTAACAATTATCGATTTTGGAC
GGACACAAAGAAAAGTTGTCAATCCTCGCTTGGGAGTTTTATATTGTTTTTCTTACCAACAGATTCGCT
GAGTCAGCAGTGGATGAAACTAGCGTTGGTATCTGGTGATATATACAACAGAAAGTTTTGTAACAATT
ATCGATTTTGGACGGACACAAAGAAAAGTTGTCAATCCTCGCTTGGGAGTTTTATATTGTTTTTCTTAC
CAACAGATTCGCTGAGTCAGCAGTG
```

**Figure 9.2.10.3:** Amino acid sequence for *Wnt16* gene.

```
MQILLLSFILFLLPTDSLQWQMKLAVALSVGIDNFRKNFNNYRFWTDTQRRKLCINYGDLPKVSVGAIRAYD
ECHIPFRWRRWNCASHAPNLSWTDSDKYPFLGRLIMSESKEAFISALFSAVGYSVSTKACQSNQLQ
SCSDVDRDTKDDSRGYYMRCHCNDDVNFVTDSKFGIDSVELNSLLRNMMYSHSRMLHNIDVRKR
TIESNKKLCAVCGVSACRISCEIPDDFRRFIGNVLLKRFDAAIKVKLKYISTRQVLMYPSPPVTNT
QLVLYKNPLYCSSVTGRRCRQKKGSESQSCNNMCCGGRYRTKERTYVKNANCFIWCCRTICQKCTFK
QKIHCK
```

**Figure 9.2.10.4:** Alignment of *Hydractinia echinata* Wnt16 protein, *Hydra vulgaris* Wnt16 protein.

<table>
<thead>
<tr>
<th>Hydractinia Wnt16</th>
<th>Hydra vulgaris Wnt16</th>
</tr>
</thead>
<tbody>
<tr>
<td>MQILLLSFILFLLPTDSLQWQMKLAVALSVGIDNFRKNFNNYRFWTDTQRRKLCINYGDLPKVSVGAIRAYD</td>
<td>ECHIPFRWRRWNCASHAPNLSWTDSDKYPFLGRLIMSESKEAFISALFSAVGYSVSTKACQSNQLQ</td>
</tr>
<tr>
<td>SCSDVDRDTKDDSRGYYMRCHCNDDVNFVTDSKFGIDSVELNSLLRNMMYSHSRMLHNIDVRKR</td>
<td>TIESNKKLCAVCGVSACRISCEIPDDFRRFIGNVLLKRFDAAIKVKLKYISTRQVLMYPSPPVTNT</td>
</tr>
<tr>
<td>QLVLYKNPLYCSSVTGRRCRQKKGSESQSCNNMCCGGRYRTKERTYVKNANCFIWCCRTICQKCTFKQKIHCK</td>
<td></td>
</tr>
</tbody>
</table>

"*" (asterisk) indicates positions which have a single, fully conserved residue. "::" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.

"." (dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix.
Figure 9.2.10.5: Alignment of Hydractinia echinata 16 protein and different Hydra Wnt proteins. **"** (asterisk) indicates positions which have a single, fully conserved residue (highlighted in green). **":"" (colon): indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. **"."** (dot): indicates conservation between groups of weakly similar properties - scoring <= 0.5 in the Gonnet PAM 250 matrix. Amino acid residues occurring in four out of five sequences are highlighted in orange.
9.3 Web sites used

http://www.stanford.edu/group/nusselab/cgi-bin/wnt/

http://www.stanford.edu/group/nusselab/cgi-bin/wnt/conservation