Dissertation
submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

Presented by

Regina Mei Mei **Teo**
Place of birth: Singapore
Oral Examination:

The Wnt Cascade and Stem Cell Fate in a Basic Metazoan

Die Wnt-Kaskade und das Schicksal von Stammzellen in einem basalen Metazoon

Referees: Prof. Dr. Werner A Müller

Prof. Dr. Monika Hassel

Acknowledgments

I thank my supervisors, Prof. Dr. Werner A Müller and Dr Uri Frank for not only giving me the opportunity to embark on this project, but also for their unfailing support and exacting tutelage throughout the course of the PhD.

I thank Dr Conrad Kunick, Technical University of Braunschweig, for a sample of the substance azakenpaullone and Dr Nicole Rebscher for the plasmid fragment of the *Hydractinia vasa.*

Within the Institute, I thank the members of my laboratory for their constant support, help and advice, especially Jasenka Guduric-Fuchs, Ibrahim Mali, Yuki Katsukura, Jördis Gramlich and Susanne Kremer. I thank Prof. Dr. Stephan Frings, for the usage of the microscopes and also members of his group for technical advice and discussions, especially Frank Möhrlen and Gabriele Gunther. I similarly thank Dr. Nicole Rebscher and the members of her group.

I thank Rajeeb K Swain and Danila Baldessari for help and discussions on the Wnt aspects of the thesis. I thank Dr Ofer Mokady (Tel Aviv University) for many discussions with respects to the microsatellite section and help in interpretation of the results.

I thank my parents and sister in Singapore and my “family” in Heidelberg: Sonia Ciarmatori, Markus Tyroller, Olivia Salazar and Tobias Odenwald, for their love, encouragement, understanding and support.

Finally, I thank my God, for creating this enriching experience for me in Heidelberg.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Abstract

The WNT signalling pathways have numerous controlling functions in embryonic development, pattern and axis formation of animals, but also play roles in sustained normal development. There are currently three known WNT signalling pathways, of which the canonical pathway is the most studied. This pathway functions not only in the establishment of the anterior-posterior axis and in cell differentiation, and is the only pathway known to play a role in stem cell fate and maintenance within many of the chordate metazoans. This work served to establish if such a role has already evolved early in metazoan phylogeny by using *Hydractinia*, a primitive eumetazoan as a model.

This work established the presence of a WNT pathway in the hydrozoan *Hydractinia*. The WNT receptor in *Hydractinia*, *He-frizzled*, has been cloned and *in situ* expression in the life cycle suggest its putative function in budding and regeneration of polyps and in the proliferation of cells during the course of the normal development of the animal. Furthermore, the *Hydractinia Frizzled(s)* is the first gene from the WNT cascade(s) that was found to be expressed in cnidarian stem cells.

Functional studies were carried out using inhibitors to a key enzyme of the canonical WNT pathway, GSK-3ß. Such an inhibition simulates the effect of the Wnt signal. Changes to colony morphology and effects on the stem cell population of *Hydractinia* were observed. Inhibitors caused ectopic formation of tentacles and heads, and also resulted in the formation of multiheaded polyps. Short treatment caused an initial high proliferation of cells followed by highly significant numbers of I-cells and a few days later, the I-cell differentiated products, namely the nerve and stinging cells. Long term exposure to the inhibitor led to slower growth of the colony, higher numbers of oogonia-like structures and an absence/lack of oocyte and eggs. All these data lend support the multiple functions the WNT pathway plays in this cnidarian and its involvement in the recruitment of stem cell derivatives from the population of I-cells.

In a separate study, an attempt to study the fate of closely related chimeric partners in *Hydractinia* was carried out using microsatellites. An enrichment protocol gave high numbers of microsatellites compared to traditional shotgun methods. There exist microsatellites within *Hydractinia*, however efforts to find a locus polymorphic enough to discriminate between two closely related kin failed.

Zusammenfassung

Wnt-basierte Signalsysteme haben zahlreiche steuernde Funktionen in der Embryonalentwicklung der Tiere. Die gegenwärtige Forschung hebt ihre Rolle bei der Etablierung von Körperachsen und der Musterbildung hervor; sie haben aber auch Bedeutung in der postembryonalen Phase des Lebens. Dies gilt vor allem für den sogenannten kanonischen Signalweg, der im Säuger bei der Einrichtung der antero-posterioren Achse beteiligt ist, weiterhin bei der Steuerung der Zelldifferenzierung und zeitlebens bei der regenerativen Erneuerung von Geweben aus Stammzellen. Dies gilt auch für andere Wirbeltiere, auf die sich die Forschung bisher konzentriert hat. Die vorliegende Arbeit hatte zum Ziel herauszufinden, ob Wnt-Signalsysteme bereits bei urtümlichen Metazoen mehrfache Rollen spielen und auch in das Schicksal von Stammzellen eingreifen. Untersucht wurde die Ontogenie von *Hydractinia echinata*, einem Vertreter der Cnidarier und damit der urtümlichsten Gruppe der Eumetazoa.

Die Arbeit bestätigt das Vorkommen eines WNT-Systems in diesem Hydrozoon. Ein WNT-Rezeptor *He-frizzled* wurde geklont und seine in-situ Expression im ganzen Lebenszyklus untersucht. Die Expressionsmuster legen eine Rolle bei der Zellproliferation, der Anlage von Polypenknospen und der Kopfbildung in Polypenknospen nahe. Weiterhin ist *Frizzled* das erste Gen einer WNT-Kaskade das in I-Zellen, das heißt in multipotenten Stammzellen und ihren noch nicht terminal differenzierten Abkömmlingen, exprimiert wird.

Es wurden funktionelle Studien durchgeführt mit Inhibitoren der GSK-3ß, einem Schlüsselenzym der kanonischen WNT-Kaskade. Gemäß Literaturangaben kann Inhibition von GSK-3 den Empfang eines WNT-Liganden imitieren. Eine solche Inhibition führt zu Änderungen der Morphologie der Polypen und in der I-Zellen-Population. Es traten ectopische Tentakel und multiple Köpfe auf. Kurzzeitige Behandlung führte zu einem Proliferationsschub in der I-Zellenpopulation gefolgt von einem Anstieg in der Zahl von I-Zellen-Derivaten, namentlich von Nervenzellen und Nesselzellen. Behandlung über längere Zeit führte auch zu einem Anstieg in der Zahl mutmaßlicher Oogonien, jedoch zur Reduktion der Zahl von Oocyten und reifen Eiern. Die gewonnenen Ergebnisse unterstützen die Annahme, dass bereits bei den Cnidarier WNT-Signalsysteme mulrtiple Funktionen haben und bei der Rekrutierung von differenzierten Zellen aus dem Reservoir der Stammzellen beteiligt sind.

In einer getrennten Studie wurde der Versuch unternommen, das Schicksal eng verwandter Partner in Chimaerenkolonien mittels molekularer Marker zu verfolgen. Als Marker wurden Mikrosatelliten gewählt. Ein Anreicherungsverfahren gab gute Ausbeuten. Es gibt Mikrosatelliten in *Hydractinia*, jedoch schlugen die Bemühungen fehl, einen polymorphen Locus zu finden, der es erlaubt hätte, eng verwandte Individuen genetisch zu unterscheiden.

General Introduction

1.1 Wnt Signalling

The Wnts constitute a large family of cysteine-rich, secreted glycoproteins involved in developmental processes including the regulation of cell fate, cell polarity, differentiation and migration, axis and pattern formation and morphogenetic movements (reviews Hsieh 2004, Wang 2004). The term *wnt* is an amalgam of *wingless* (*Wg*) and *int*. *Wg* is required for the formation of parasegment boundaries and maintenance of *engrailed* expression in adjacent cells for *Drosophila* and *int*, the proto-oncogene activated by the integration of mouse mammary tumour virus in mammary gland tumours (Sharma and Chopra 1976, Nusse and Varmus 1982, Cabrera et al., 1987, Rijsewijk et al., 1987). All Wnts encode a signal sequence at the N-terminus of the protein, followed by 23 or 24 cysteine residues with conserved spacing, suggesting that folding of the Wnt protein is dependent on formation of multiple intracellular disulfide bonds (Miller 2001). Several Wnt proteins are also palmityolated on a conserved cysteine residue (Willert et al., 2003).

Based on experiments that ectopically expressed Wnt proteins in mammary epithelial cells and on secondary axis induction in *Xenopus* embryos, Wnts have been loosely divided into two classes: the Wnt-1 class comprising the mouse Wnt-1, -3a and -7a and the *Xenopus* Xwnt-1, -3a, -8a and -8b. Mis-expression studies showed that these Wnts are capable of transforming mammalian cells in culture (Wong et al., 1994) and of inducing a secondary axis when ectopically expressed in the future ventral side of early frog embryos (McMahon and Moon 1989). The proteins from the Wnt-5a class function differently from those in class Wnt-1, and are instead able to influence cell adhesion and cell migration (Du et al., 1995; Torres et al., 1996, Heisenberg et al., 2000). This latter class includes the mouse Wnt-4 and -5a genes and the *Xenopus* Xwnt-5a and -4. Chimeric proteins generated by combining elements of Xwnt-8 (a Wnt-1 class protein) and Xwnt-5A (a Wnt-5a class protein) and used for axis induction experiments in frog embryos, showed that the different activities of the two Wnt classes are determined by the C-terminal portion of the proteins (Du et al., 1995).

Wnts bind to receptors of the Frizzled family, named after the *Drosophila* tissue polarity gene *frizzled*. Structurally the Frizzled receptors have an extracellular Wnt-binding, cysteine-rich domain (CRD), comprising 10 conserved cysteines in

vertebrates, seven hydrophobic domains that are predicted to form transmembrane α-helices spanning regions and an intracellular C-terminal tail, which is not well conserved (Huang and Klein 2004). The presence of the seven transmembrane-spanning domains suggests that Frizzled proteins are related to the class of G-protein-coupled receptors and that the Wnt signals are probably transmitted through receptor coupling to heterotrimeric G-proteins as in the other members of this receptor family (Huang and Klein 2004). At the C-terminal, a motif (KTXXXW) located two amino acids after the transmembrane region, is highly conserved in all Frizzleds and this is essential for activation of the Wnt/β-catenin pathway (Umbhauer et al., 2000).

The Wnt proteins signal through both autocrine (Shimizu et al., 1997, Etheridge et al., 2004) and paracrine pathways (Christiansen et al., 1996, Shimizu et al., 1997) and the pathways have been implicated in diverse biological events, especially related to embryonic development, tumourigenesis and neurodegenerative diseases. There is an apparent mutually exclusive activation of the different pathways. The Wnt-1/Wg class proteins activate the canonical Wnt/β-catenin pathway whilst the Wnt/Ca^{2+} signalling pathway is activated by the Wnt-5a type ligands. This latter pathway is sometimes referred to as non-canonical Wnt signalling (Kuhl et al., 2000).

There are three currently known Wnt signalling pathways (Fig. 1):

1. Canonical Wnt/β-catenin pathway, where the stabilization of cytosolic β-catenin and its subsequent accumulation in the nucleus leads to activation of target genes (reviews: Huelsken and Behrens 2002, Lustig and Behrens 2003).
2. Wnt/polarity, or planar polarity pathway (PCP), where the activation of the small GTPase RhoA and subsequently that of Rho-associated kinase (ROCK) and Jun N-terminal kinase (JNK) leads to oriented cytoskeletal rearrangements i.e., polar arrangement of cuticular hairs in *Drosophila* or affects convergent-extension movements in *Xenopus* (Sokol 2000, Huelsken and Behrens 2002, Povelones and Nusse 2002).
3. Wnt/Ca^{2+}, where the modulated intracellular release of Ca^{2+} (Slusarski 1997), leads to the activation of Ca^{2+} sensitive enzymes like protein kinase C (PKC), calcium-calmodulin dependent kinase II (CamKII, Kuhl et al., 2000) and calcineurin (Saneyoshi et al., 2002) This pathway affects possibly convergent extension movements (Wallingford et al., 2001), ventralisation (Saneyoshi et al., 2002) and tissue separation during gastrulation in *Xenopus* (Winklbauer et al., 2001).

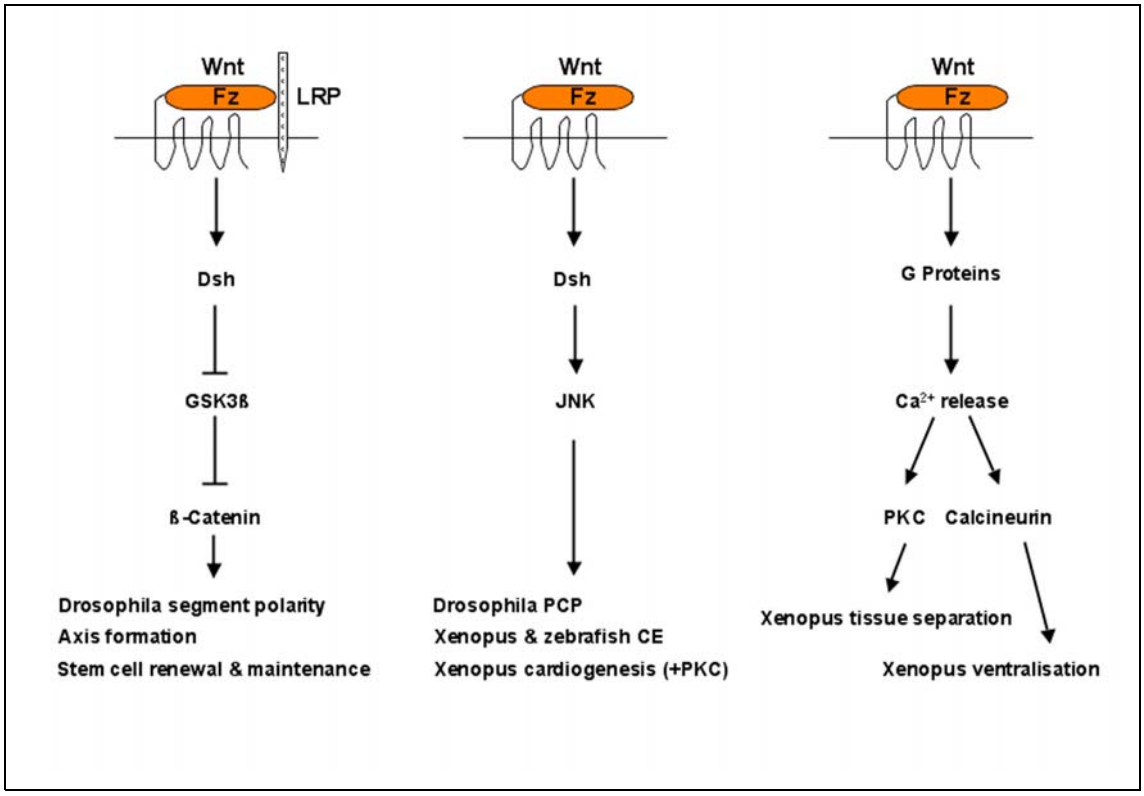

Figure 1. The known Wnt signalling pathways, (Left) Best studied and commonly known as the canonical pathway, known especially for its role in cancer and axis formation. (Middle, right) The non-canonical pathways, where induction gives phenotypes exclusive from that observed for the canonical. CE = convergent extension, Dsh = Dishevelled, Fz = Frizzled, GSK = glycogen synthase kinase, JNK = jun N-terminal kinase, LRP = low-density lipoprotein receptor-related protein, PCP = planar polarity pathway, PKC = protein kinase C.

The specific activation of any of these pathways is usually dependent on the specific Wnt-Frizzled combination (see review in Wodarz and Nusse 1998, Winklbauer et al., 2001), although Carron and co-workers (2003) have shown that in the absence of a Wnt ligand, dimerisation of the Xfz3 and Xfz7 receptors alone can possibly transduce a signal along the canonical Wnt/β-catenin pathway.

It was the discovery of the first *Wnt* gene, mouse *Wnt-1* that lead to the growth in research in the 1980s to the potential role Wnt plays in cancer. Growing evidence indicate that aberrant Wnt signalling leads to tumourigenesis (review Malliri and Collard 2003) and cancer (Howe and Brown 2004). Insofar, most evidence indicates the canonical pathway playing a role in the development of several human cancers (Austin 1997, Reya 2003). Usually tumour cells show an accumulation of β-catenin in the cytoplasm and in the nucleus, an event that arises when there are mutations in β-catenin or any other components within this pathway that block β-catenin phosphorylation and its subsequent ubiquitination. Hence β-catenin is not targeted for

degradation by the proteasome. Its accumulation and entry into the nucleus and association with the LEF/TCF group of transcription factors eventually leads to the transcription of genes (Peifer and Polakis 2000, Willert et al., 2002) that can cause tumours, e.g. of *c-myc*, a gene involved in the proliferation of cells. Concurrently there is support implicating the canonical Wnt signalling pathway in the proliferation of stem cells (Willert et al., 2003). Since cancer is the result of deregulated proliferation and suppression of cell death mechanisms, in order for oncogenic mutations to induce cancer, such dysfunctions must occur in dividing cells and the mutations passed on to progeny cells (usually somatic cells). Such a pool of dividing cells is the stem cells and when the balance of control between proliferation and differentiation is disturbed, coupled with a loss of apoptotic function, abnormal cell number population occurs and eventually also tumour growth, benign or malignant.

1.2 Stem cells

Stem cells are undifferentiated cells that can produce daughter cells remaining either as stem cells (a process called self-renewal) or are committed to a pathway that leads to differentiation (review Raff 2003). This recent interest in stem cells comes from the hope it provides as a source of therapy in degenerative diseases or amputation cases, and perhaps even cancer.

In the mammalian system where stem cells have been comprehensively studied, typically there are three potential sources of stem cells, the embryonic stem (ES) cells, which are cells obtained from the inner cell mass of the blastocyst, the embryonic germ (EG) cells, derived from the primordial germ cells of post-implantation embryos, and adult stem cells. The categories by which mammalian stem cells are defined, is dependent on the types of progeny they give rise (Morrison et al., 1997). Based on this potential, stem cells are classified as follows (Wagers and Weissman 2004):

1. Totipotent, having the ability to give rise to all embryonic and extra-embryonic cell types, e.g., the zygote and the blastomeres at the 2-cell stage.
2. Pluripotent, able to give rise to all cell types of the embryo proper, but not the extra-embryonic tissues, e.g., the ES cells.

3. Multipotent, where the progeny of these stem cells are of multiple differentiated cell types, but all within a particular tissue, organ, or physiological system, e.g., the haematopoietic stem cells.
4. Oligopotent, when its progenitor cells are able to produce a few different types of mature cells, e.g., the lymphoid precursor in haematopoiesis.
5. Unipotent, able to give rise to only one mature cell type, e.g., stem cells of the keratinocytes in skin, or spermatogonia into spermtocytes.

It must be emphasised that this subdivision and these definitions are applicable only to mammals and amniotes, since extraembryonic structures are not common in animal development. Moreover, linguistically pluripotent (Greek : pluri = many) and multipotent (Latin: multi = many) are synonymous. Therefore, in this work, no difference will be made between the two.

The cells of the mammalian embryo within the first three days of development are currently accepted as cells with totipotent abilities. After four days, the cells start to specialise and form the blastocyst. The outer cell layer of the hollow blastocyst gives rise to the trophoblast and eventually to the placenta and other tissues that are required for the support and development of the embryo, while the inner cell mass continues to divide and differentiate into the various tissues and cell types of the organism. Thus, cells from the inner cell mass are pluripotent.

The haematopoietic stem cells (HSCs) are currently the best characterised stem cell population that are multipotent. For example, one haematopoietic stem cell not only produces progeny that include HSC (self renewal), but also blood cell-restricted oligopotent progenitors of all cell types and elements (e.g. platelets) that are normal components of the blood (Müller 1996, Wiessman 2000). The clonal common myeloid progenitor is oligopotent as it can give rise to blood granulocytes, monocytes/macrophages, red blood cells, platelets, basophiles, eosinophiles and dendritic cells, but not to the T lymphocytes, B lymphocytes, or natural killer (NK) cells. These are progenitors of the second stem cell lineage, the lymphoid precursors.

The most fundamental stem cell lineage is the germ line, conveying life from parents to their progeny. Studies into model organisms of mechanisms responsible for germ cell specification have been divided into two modes: preformation i.e., these germ cells are set aside early in embryogenesis, and their differentiation into germ cells at this stage are ascertained by localized maternally inherited determinants; these

particulate markers are the P-granules in *Caenorhabditis elegans*, polar granules in *Drosophila*, or "germ plasm" in the egg of *Xenopus*. In other organisms like annelids and mammals, these cytological markers are termed nuage, which is a fibrous material surrounding the nucleus. The second mode of germ line determination is inductive i.e., when germ cells are differentiated later in development, and are brought about by inductive signals from the surrounding tissues (review Extavour and Akam 2003). For example, in mouse, the germ cell lineage is established at approximately 7.2 days post coitum (Ginsburg et al., 1990, McLaren 2000). In mammals, these primordial germ cells are undifferentiated precursors that transiently exist in the yolk sac and remain in this location until they migrate into the gonad and become irreversibly committed as germ cells. Cells other than primordial germ cells are known as somatic cells and these cells later differentiate into tissues and organs.

Molecular markers of the germ line are products of several genes such as *vasa* (*vas*), *nanos* (*nos*) and *oct4*. These products are present as mRNA-transcripts or translated proteins. The products of these genes may be bound to the above mentioned particulate structures, but also occur in the cytosol outside these structures. Germ-line restricted products in hydroids are detailed later.

1.3 Interstitial stem cell lineage in Cnidaria

Historically, the presence of cells preserving embryonic features in adult organisms and serving as a resource for cell replenishment were first found in Hydrozoa (Kleineberg 1872 quoted in Martin and Thomas 1981) and planarians (Keller 1894, in Peter et al. 2004). These became known in Hydrozoa as interstitial cells, which have been abbreviated to I-cells, and in the planarians as neoblasts. Thus studies in I-cells were paradigmatic for stem cells in general until the focus switched exclusively to stem cells in mammals.

The hydrozoan stem cell system has been investigated from ontogeny (Martin and Archer 1997, Müller 1967) and proliferative capacity (Plickert et al., 1988) to totipotency abilities (Müller et al., 2004a). Based mainly on descriptive studies, I-cells were proposed to be pluripotent or even totipotent by several authors (Davis 1970, Haynes and Burnett 1963, Sacks and Davis 1979). The occurrence of apparently intermediates between I-cells and differentiated cells prompted this notion. These arguments were subsequently considered invalid by subsequent investigators. The developmental potencies of the I-cells remained a matter of debate for decades since

potencies appear to differ among the various genera and species. For instance, in *Hydra* the potencies were found to be more restricted (Bode 1996) compared to *Hydractinia* (Müller et al., 2004a). This will be expanded later.

Most current knowledge on interstitial cells is from studies on the freshwater hydroid, *Hydra* (review Bode 1996). It is assumed that hydroids of the genus *Hydra* have three stem cell lineages: the ectodermal and endodermal epithelial lineages which are unipotent, and the multipotent interstitial lineage which gives rise to four classes of differentiation products: male or female gametes, two types of secretory cells (mucous and gland cells), four types of stinging cells and two types of nerve cells (sensory and ganglionic).

Of the genes known to be expressed in the interstitial cells of cnidarians are the *Cnos1* and *Cnos2*, *nanos*-related genes known to be required for the maintenance of germ cell lineages in *Drosophila* and *Caenorhabditis elegans* (Mochizuki et al., 2000), *Cnvas1* and *Cnvas2*, *vasa*-related genes known to be involved in the germ cell formation in higher metazoans (Mochizuki et al., 2001), serum response factor homologues *HvSRF* in *Hydra vulgaris* and *HeSRF* in *Hydractinia echinata* (Hoffman and Kroiher 2001) and *Hyzic*, the *Hydra* homologue of the zinc finger transcription factor zic/odd-paired (Lindgens et al., 2004). *Hyzic* is also expressed in nematoblasts, which are differentiating products of I-cells. In *Podocoryne carnea*, *Cniwi*, a *Piwi*-like gene that represents essential stem cell genes in multicellular organism, is also expressed in somatic cells derived from transdifferentiating smooth/cross striated muscle cells (Seipel et al., 2004a).

The germ stem cell lineage in the freshwater hydrozoan *Hydra oligactis* has similarities to that of the mammalian system in that primordial germ cells are not set aside early in embryogenesis. Germ cell specific genes in cnidarians include those that are also expressed in the true interstitial cells (see above section), with the exception of *Hyzic*. Within *P. carnea*, two orthologues of the *nanos* gene, *Pcnos1* and *Pcnos2*, have also been isolated and expression of both genes was eventually restricted to that of the mature gonads (Torras et al., 2004). Recently, two homologues of the CCAAT/enhancer binding (C/EBP) protein and the Maf protein of the leucine zipper transcription factor subfamily have been reported to be localised in the mature gonads of both sexes, restricted to the oogonia and the spermatogonia of *P. carnea*, respectively (Seipel et al., 2004b). The *Cniwi* gene is also expressed at low levels in the gonads of *P. carnea* (Seipel et al., 2004a).

1.4 The study organism: *Hydractinia echinata* (Fleming, 1828) (Metazoa: Cnidaria: Hydrozoa: Athecata: Hydractiniidae)

The marine colonial hydroid *Hydractinia echinata* (Fig. 2), is an athecate cnidarian that frequently lives in commensalism with pagurid hermit crabs inhabiting gastropod shells (*Littorina* spp. or *Buccinum* spp.) (Frank et al., 2001). Its distribution is limited to the northern coasts of Europe located in rocky pools and sandy shores and confined mainly to the smaller shells of these hermit crabs.

In the wild, the mature colony comprises four distinct zooids: gastrozooids for feeding, gonozooids for reproduction, dactylozooids and tentaculozooids, probably for defence. This gonochoristic hydroid lacks free-living medusae for its reproductive stage. Instead, its life cycle begins with the synchronised release of gametes from gonophores. These are structurally reduced ball-shaped medusae that are produced by gonozooids (sexual polyps), remain sessile and serve as gonads (Figs. 2a-b). The gametes are released into the surrounding waters, where fertilization takes place. Embryonic development takes approximately 72 hours till the planula larvae are fully competent for metamorphosis (Plickert et al., 1988).

In cnidarians, as with many marine invertebrates, the cues triggering metamorphosis is dependent on the external environment instead of differential hormonal levels within the organism (Müller and Leitz 2002). After attaching to a suitable substratum, the cone-shaped planula larvae are triggered to metamorphosis. This process is probably initiated by the presence of bacteria from the genera *Alteromonas* and *Pseudoalteromonas* covering these mollusc shells. Experimental data suggests that the metamorphosis-inducing cue comes either from the outer bacterial wall or from the fimbriae produced by the bacteria (Müller and Leitz 2002).

The metamorphosis into a primary polyp and subsequent growth of the colony takes place initially via extension of its gastrovascular canals (stolons), with carefully spaced, controlled growth of new gastrozooids. After an initial growth period, and depending on the health of the colony, gonozooids develop.

H. echinata has already served as model organism in the study of developmental biology and mechanisms of allorecognition (review Frank et al., 2001). Colonies are easily maintained within the laboratory, cultured at temperatures of an average 18^{o}C. Daily feeding with *Artemia* nauplii allows for an almost daily release of gametes, with the spawning process controlled using a 15-9 hour light-dark

regime. Collection of the fertilised eggs, and subsequent developmental stages can be manipulated easily for further experiments.

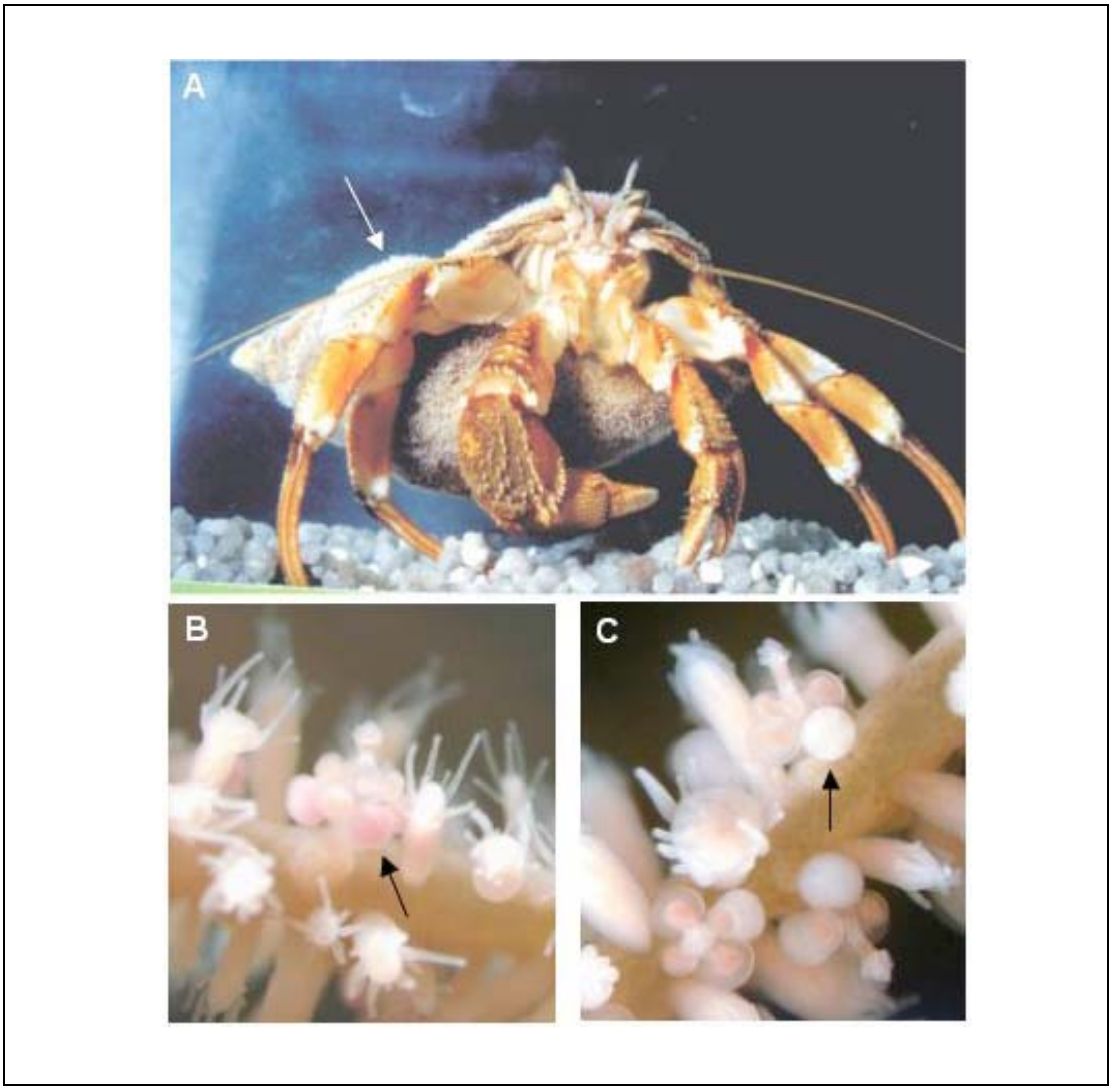

Figure 2. *Hydractinia echinata*. (A) Colony (arrowed) normally lives in commensalism with hermit crabs. Photo by Rolf Lange (B) Female colony. (C) Male colony. Colonies have separate sexes with medusae reduced to gonophores that function as gonads (arrowed). These remain sessile, and gametes are released into surrounding water when mature. Photos by Yuki Katsukura.

1.5 Interstitial stem cell lineage in *Hydractinia echinata*

As with most hydroids, the interstitial cells (I-cells) of *H. echinata* first occur in the endoderm of the preplanula (Munck 1986), probably within the first 12 hours of embryogenesis (Hoffman and Kroiher 2001), with characteristic morphology at 25 hours after fertilization. With the onset of metamorphosis, the I-cells migrate across the mesoglea and reside in clusters of three of four cells (Van de Vyer 1964) within the interstitial spaces of the ectoderm. Within the mature colony, the I-cells are

located between the lower and upper epithelium, residing often along the endodermal canals (Fig. 3). However, the youngest tissues (at the periphery of the colony) are often devoid of I-cells. Migration of I-cells occurs within the interstices of these epithelial layers (Müller et al., 2004a).

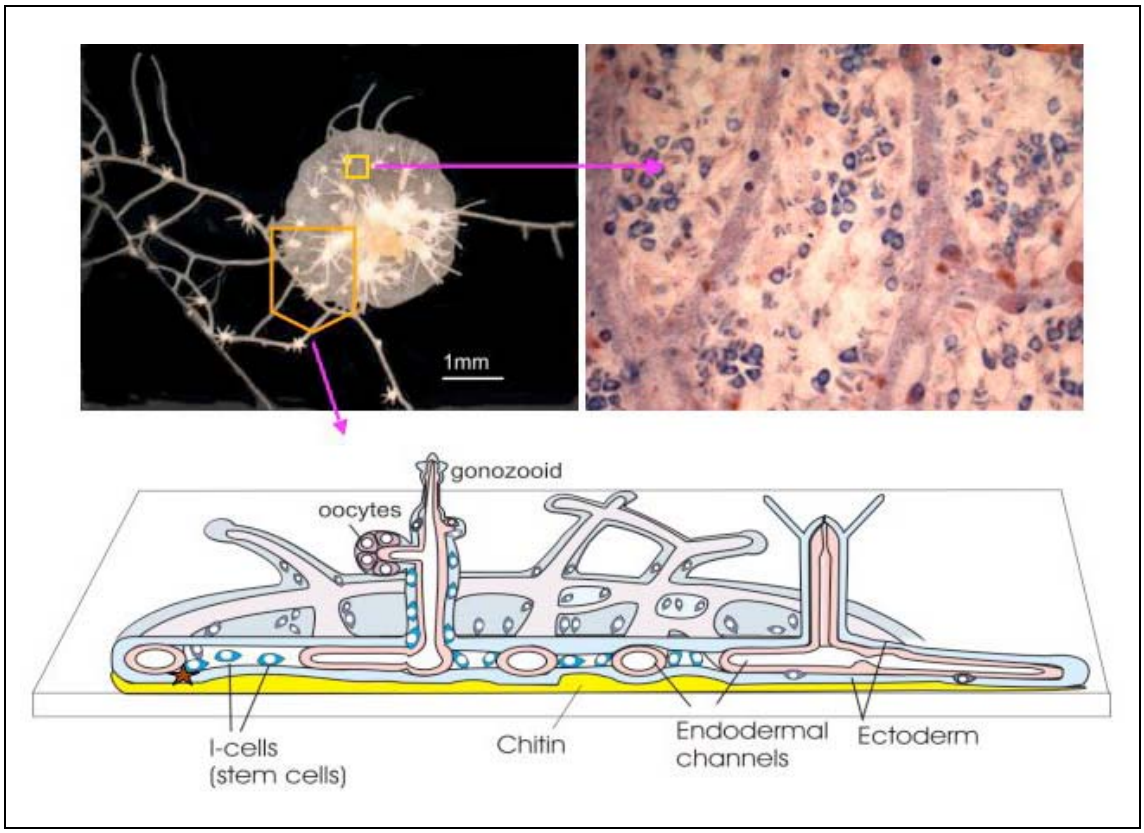

Figure 3 (Müller et al, 2004a). Interstitial cells in *H. echinata*. (Top left) Overview of colony. (Top right) Whole mount preparation of stolonal mat stained with May-Grünwald and Giemsa stains. Cytoplasm of interstitial cells stained strongly due to high concentration of ribosomes. (Bottom) Schematic representation of colony indicating I-cells migrating between interstices of the epithelia.

I-cell elimination studies in *H. echinata* (Müller 1967, Müller et al. 2004a) resulted in a lack of feeding ability from the treated colonies and immobility. With time also death occurs if there were no introduction of new I-cells. Introduction from a histocompatible donor however, led not only to colony recovery i.e., the restoration of the ability to capture prey by the use of cnidocytes (nematocytes) and engulfment of food, but also the subsequent takeover of the recipient into the phenotype of the donor, down to the germ line. Hence the I-cells of *H. echinata* are potentially able to give rise to cnidocytes (nematocytes), and nerve cells but also epithelial and germ cells (Müller 1967).

In the classical understanding of the I-cell potential within hydroids, based on comprehensive studies in *Hydra* (review Bode 1996), the I-cell lineage is a multipotent stem cell lineage that only gives rise to four classes of progeny (listed above). In contrast, recent studies by Müller and co-workers (Müller et al., 2004a) showed that these I-cells have totipotent capacities, by their ability to traverse into the epithelial stem cell lineage. According to the *Hydra* community, the epithelial stem cell lineages are unipotent and these are separate from the interstitial stem cell lineage. However, Müller and coworkers showed that in colonies of *H. echinata* depleted of I-cells with akylating agents and introduced with I-cells from a histocompatible donor, the recipients eventually phenotypically transformed to the phenotype of the donor, down to the sex of the colony. Experiments with BrdU labelled migratory cells from the donor also showed the presence of BrdU stained nuclei within epithelial cells, which can only come from the donor I-cells that have migrated to these sites and differentiated, as epithelial cells by definition are stationary, due to their adherence to the basement membrane and to neighbouring epithelial cells by adherens junctions. Genotypically, whether the recovered animal is a chimera or has completely converted to the donor, can only be answered with the use of polymorphic markers like microsatellites.

1.6 Wnt signalling in Cnidaria

Knowledge of the presence of genes related to the Wnt signalling pathway in Cnidaria came with the isolation of a homologue of *β-catenin* in *Hydra* (Hobmayer et al., 1996). Discovery of other Wnt related genes like the Wnt receptor *Frizzled* in *Hydra* (Minobe et al., 2000) and subsequently *HyWnt*, *HyDsh*, *HyGSK3* and *HyTcf* (Hobmayer et al., 2000), together with *in situs* showing expression at regions of new bud formation and head regeneration established the presence of the canonical Wnt pathway within a basal eumetazoan. This same study further confirmed the commonly known function of the canonical pathway involved in axis formation when the authors injected mRNA of the *Hydra* homologue of *β-catenin*, *Hyβ-cat,* into ventral blastomeres of *Xenopus* at the eight-cell stage, and induced a secondary axis. In addition, in a descriptive study on the role of nuclear β-catenin in embryogenesis of the anthozoan *Nematostella*, a role for the canonical pathway in the determination of germ layers was suggested (Wikramanayake et al., 2003).

A further hint as to a role of the Wnt-related pathways in axis formation comes from studies involving chemicals. Tumour-promoting phorbol esters, well known activators of PKC, induced head regeneration in *Hydractina* polyps also at the basal end of the body column and diacylglycerol (DAG), another chemical shown to stimulate the activity of PKC, which in turn can inhibit GSK-3β activity (Cook et al., 1996, Chen et al., 2000), caused ectopic head formation along the body column of *Hydra* (Müller 1989). Another chemical, alsterpaullone, shown to be a GSK-3β inhibitor, also induced ectopic head and tentacle formation along the body column of *Hydractinia* polyps and along enlarged "stolonal" buds that subsequently developed polyps (Müller et al. 2004b).

The isolation of phospholipase C-beta cDNAs from *Hydra* (Koyanagi et al., 1998), a protein already shown to be involved in the Wnt/Ca^{2+} pathway, suggests the probability of such a pathway within cnidarians. Presence of the *Hydra* PKC genes augments this and expression of the *Hydra* PKC gene, *HvPKC2*, at the same location as the *HyWnt* (Hassel et al., 1998) supports this, and suggest possible crosstalking between the two pathways (Müller et al., 2004b). Currently, no homologous members from the NF-AT family transcription factors have been isolated in cnidarians. To date, no known homologues along the PCP pathway has been isolated.

Questions

Assuming that the evolutionary function of the Wnt pathways are conserved throughout metazoan phylogeny, and especially so within the phylum Cnidaria, then the canonical Wnt pathway has the potential of not only playing roles in patterning in *Hydractinia* embryos and axis formation within the adult, but also in renewal and proliferation of the I-cell lineage. I investigated this question by:

1. Using a gene from the canonical pathway and documented the expression patterns during embryogenesis and development, and especially those associated with the I-cell lineage. I chose *frizzled* because expression of Frizzled receptors indicates responding cells.
2. Using alsterpaullone, a specific blocker to GSK-3β, I investigated the effects on the I-cell fate, especially within the true I-cell population, the precursors and terminally differentiated products of these I-cells, namely nematocytes, nerve cells and germ cells.

Material and Methods

2.1 Materials

2.1.1 Technical equipment

Centrifuge laborfuge 400R	Heraeus
Centrifuge 5415C	Eppendorf
Cryostat Leica CM 3050 S	Leica
Digital camera Color view	Soft imagining system
Fluorescence microscope Axiovert 200	Zeiss
Gel documentation system with BioDocAnalyze software	Biometra
Hybridisation oven / shaker S1 20H	Stuart Scientific
PCR machine Mini Cycler	MJ Research
PCR machine TGradient	Biometra
pH-meter 109	Corning
Power supply E844 and E443	Life Technologies
Power supply PS 3002	Consort
Sonifier B15	Branson
Sequencing System Model S2	GIBCO BRL, Life Technologies
Thermoblock TB1	Biometra
Ultracentrifuge L70	Beckman
UV Transilluminator TFX-35LC	Vilber Lourmat
UV/VIS Spectrophotometer Lambda 2S	Perkin-Elmer

2.1.2 Chemicals

Agarose	Roth,
APS (Ammoniumpersulphate)	Serva
Bacto Tryptone	Difco
BCIP (5-bromo-4-chloroindolylphosphate sodiumsalt)	Serva
Bind-Silane	Pharmacia Biotech
BlueSlick	Serva

BSA (bovine serum albumin)	Roth
Bromophenolblue	Serva
Carbencilin,	Serva
Chlorophorm	Fluka
Dextransulfate	Sigma
Diethylpyrocarbonat (DEPC)	Sigma
Dimethylformamide (DMF)	Merck
Dimethylsulfoxide (DMSO)	Merck
Dithiothreitol (DTT)	SMART™ Clontech
Ethidiumbromide	Fluka
Formaldehyde	Merck
Formamide	Roth
Giemsa	Merck
Glycerol 99,5%	Roth
Guanidinium isothiocyanate	Roth
Heparin sodium	Serva
Herring sperm DNA	Roche
IPTG (isopropylthio-β-D-galactoside)	Roth
Isoamilalcohol	Riedel de Haen
Lithiumchloride	Merck
Magnesium chloride	J.T.Bacer
Mannitol D	Sigma
May-Grünwald	Merck
Mercaptoethanol (2-)	Merck
Methylene blue	Sigma
MOPS (morpholinopropane sulfonic acid)	Serva
NBT (nitro blue tetrazolium-chloride)	Roth
Phenol	Roth
Select agar	Gibco BRL

Select yeast extract	Gibco BRL
Sodium acetate	Merck
Sodim azide	Serva
Sodium chloride	Merck
Sodium citrate	Merck
TEMED (Tetramethylethilene diamine)	Serva
TRIS	Roth
Triton X-100	Serva
TWEEN-20	Serva
X-Gal	Roth
Xylene cyanol	Serva

2.1.3 Enzymes

Enzyme	**Activity**	**Company**
Powerscript reverse transcriptase	200U/µl	Clontech
RevertAide H minus M-MuLV reverse-transcriptase	200U/µl	MBI Fermentas
SP6 RNA polymerase	20 U/µl	MBI Fermentas
T3 RNA polymerase	20 U/µl	MBI Fermentas
T7 RNA polymerase	20 U/µl	MBI Fermentas
T4 DNA ligase	1 U/µl	MBI Fermentas
Taq DNA polymerase	1 U/µl	MBI Fermentas
Restriction enzymes: EcoI (EcoRV), Eco52I, EcoRI, HindIII, NcoI, NotI, SacI, EcoRV	10 U/µl	MBI Fermentas
Ribonuclease Inhibitor	50 U/µl	MBI Fermentas

2.1.4 Vectors

pBluescript II SK+	Stratagene
pGEM-T Vector	Promega

2.1.5 Bacteria

Echerichia coli XL-1-Blue	Strain for pBluescript, and pGEM-T vector

2.1.6 Kits

MagneSphere® Magnetic Separation Products	Promega
NucleoSpin® Plasmid Mini Kit	Macherey-Nagel
NucleoSpin® Extract	Macherey-Nagel

2.1.7 Other molecular biology products

Product	Concentration	Company
ATP	100mM	MBI Fermentas
dATP, dCTP, dGTP, dTTP	100mM	MBI Fermentas
Digoxigenn (DIG) RNA labelling mix		Roche
Anti-DIG-AP, Fab fragments		Roche
dNTP mix	10mM	MBI Fermentas
NTP mix	10mM	MBI Fermentas
Biosizer ™V		Hybaid
GeneRuler 100bp Ladder Plus		MBI Fermentas
RNA-Marker low range		MBI Fermentas
RNA 2x Loading Buffer		MBI Fermentas
PCR buffer 10X (750mM Tris-HCl pH 8.8, 200mM $(NH_4)_2SO_4$, 0.1%Tween		MBI Fermentas
Chromatography paper	3 MM	Whatman®

Hybond-N Nylon Membrane		Amersham Biosciences
Nytran Super Charge nylon transfer membrane	0.45 µm	Schleicher & Schuell
Water for molecular biology		Fluka (Sigma)

2.1.8 Oligonucleotides

2.1.8.1 Specific Oligonucleotides

Oligonucleotides were designed using the software located at: http://frodo.wi.mit.edu/primer3/primer3_code.html

FzDegF	ATG AGY GCN TCI ATH TGG TG
FzDegR	GT YTT NCC IGA CCA DAT CCA
FzRACEF1	TTG CTG TAC TTG CAA TGA ATC A
FzRACER1	TAA GCC AAG CAC TTA TCC AAG A
FzRACER2	GGC AGT TGA TAA CAT GCA GAC A
SAULA	GCG GTA CCC GGG AAG CTT AA
SAULB	GAT CCC AAG CTT CCC GGG TAC CGC
Biotin-$(CA)_{12}$	Biotin-CAC ACA CAC ACA CAC ACA CAC ACA
DIG-$(CA)_{12}$	DIG- CAC ACA CAC ACA CAC ACA CAC ACA
µsat1HF	TAT CCA ACG TCG CAT GCT CAT C
µsat1HR	GGG GGC TGT TAG GGT ATG GTG A
µsat1KF	CCA TTG ACT TGC CTC AGA TAC A
µsat1KR	GTG TGA ATC AGT TGC CAT CG
µsat2BF	CGA CCG CAA AAC AAA TTC ACA G
µsat2BR	AAC CAA TGT GGG AGC AAG GTG
µsat2GF	CAG AAA GCG TGG ATG AGG TAA C
µsat2GR	TGT AAA ACG ACT CCC AGA GAG C
µsat2HF	ACG ATG CGA TAT GTC ACA GC
µsat2HR	AAC TGA TCT CCC GCA TAA AAA G

μsat 2KF	GCA AAC ACT CAC ATA CGC TCA C
μsat2KR	CGA TCA GTA ACC TCA TGG CTC
μsat2PF	ATA AGC AGG CGT AGC GAG CAC T
μsat2PR	CCG GGG GAT CAG CAA CAT TA
μsat5F	TGA GAA TCT TCG CTG TGA G
μsat5R	AGT AGG GTG GGA GTG GTG A
μsat19F	CGA CTC CTC AAG CAG CAA CT
μsat19R	TGG AGG ATG CAG GTC TTG A
μsat30F	GAT ATC CAA CGT CGC ATG CTC ATC
μsat30R	GGG GCT GTT AGG GTA TGG TGA GTG
μsat48F	AAC ACC CCT GCC GCA CAA ATC
μsat48R	GCG TCG CCC AAG TGA GCA TAA C
μsat52F	ATC TTT GCA CAG AGA GGC AAA C
μsat52R	AGC TTC ACC TCT GCC CTT AC
μsat58F	ACT TAC TGT CTG TAC CCA AAT GC
μsat58R	GCT CCA TAT GGC CGA GGT
μsat59F	TTG ACT TGC CTC AGA TAC AG
μsat59R	CTG TAT AGC AAG TGT GAG TC
μsat66F	GTC TGT ACC CAA ATG CCG CAA CC
μsat66R	GAT CAG CGA CGC CAT GCC AGC
μsat67F	AGT CTC CGC CTA GAA GGT TTG C
μsat67R	TGA AGC GCC CAA CGA TTC
μsat81F	GGT GTG TAC GCT GAT ACA CTT ATG
μsat81R	CTG GAC CTC TCG TCT CAC C
μsat128F	TGA GTG CGG ACA CAG AAA GCG
μsat128R	ACA ACT TCC GCC TAG CAC CGC
μsat133F	TTC GGA CTG AAG TGT CAT TAA GTC
μsat133R	GAT CGA GAA TTG GCT GGC AAG

2.1.8.2 Standard Oligonucleotides

M13/ 22-mer sequencing primer	GCC AGG GTT TTC CCA GTC ACG A
M13 reverse/ 24-mer sequencing primer	GAG CGG ATA ACA ATT TCA CAC AGG
SP6/ 18-mer promotor primer	ATT TAG GTG ACA CTA TAG
T3/24-mer promotor primer	GCG CGA AAT TAA CCC TCA CTA AAG
T7/20-mer promotor primer	TAA TAC GAC TCA CTA TAG GG
SMART II	AAG CAG TGG TAA CAA CGC AGA GTA CGC GGG
UPM long	CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA ACA ACG CAG AGT
UPM short	AAG CAG TGG TAA CAA CGC AGA GT

2.2 Methods related to RNA

2.2.1 Total RNA isolation from tissue by acid Phenol-Guanidium thiocynate-chloroform extraction method

Approximately 25-50 mg of tissue was lysed in 1 ml of GNP mix (1:1:0.1 4M Guanidium thiocynate + 0.7% ß-mercaptonal:phenol:sodium acetate). 200µl chloroform was added and vortexed vigorously for one minute. The samples were incubated on ice for 20 minutes. Centrifuge at 4^{o}C, 20000g, 20 minutes. The aqueous layer was transferred out and 250µl ice-cold isopropanol and 250µl 1.2M sodium chloride/0.8M sodium citrate was added to it. It was incubated at room temperature for 30 minutes, vortexed 30 seconds and then centrifuged at 4^{o}C, 12000g, 10 minutes. The pellet was air dried for 10 minutes. 400µl lithium chloride was added to the pellet and incubated for five minutes at room temperature. These were centrifuged at 4^{o}C, 5000g, 10 minutes. The pellet was dissolved in 250µl of Solution D + 0.7% ß-mercaptonal mix, with intermittent heating at 65^{o}C, and some vortexing, if needed. The samples were spun down, 250µl of ice cold isopropanol added to it and mixed well by briefly vortexing. This was incubated for at least 30 minutes at -20^{o}C and then centrifuged at 4^{o}C, 10000g, 15 minutes. The pellet was washed with 300µl of 70% ethanol in DEPC-water and subsequently incubated for 15 minutes at room temperature. Another round of centrifuge at 4^{o}C, 10000g, 10 minutes and the pellet air dried for approximately 10 minutes at room temperature. The RNA was dissolved

in 20-30µl of RNAse-free water, with heating of the pellet at 60°C, and intermittent vortexing for five seconds. This was repeated at least twice. RNA concentration was determined at A_{260} ($1A_{260}$ ssRNA = 40 µg/ml) and RNA integrity was checked on a formaldehyde gel.

2.2.2. Ascertaining quality of RNA using formaldehyde gel

A 1.4% agarose gel with formaldehyde and MOPS (1.4 % Agarose; 1x MOPS; 7.4% formaldehyde) was cast. RNA samples were mixed with 1x loading buffer, heated at 70°C for 10 minutes to denature the RNA, spun down and put on ice. The gel was ran (running buffer: 1x MOPS, 7.4% formaldehyde) at maximum 100V, for half an hour and the gel viewed under UV transilluminator. For checking RNA integrity, a minimum of 500ng was loaded on the gel. For Northern hybridistions, a minimum of 7 µg of total RNA was separated on the gel.

2.2.3 DIG-RNA labelling using run-off transcription

The RNA probe was prepared by *in vitro* transcription reaction using a fragment of the *He-frizzled* gene cloned into the pBluescript vector as a template. For the synthesis of the probe with T3 polymerase the vector was cut with Hind III, and for the T7 reaction with EcoRI. The digested plasmid was eluted from the 1% agarose gel using the NucleoSpin® Extract kit. The transcription reaction contained 1µg of template, 1x RNA labelling mix, 1x transcription buffer, 80U of T3 or T7 polymerase, 80U RNAase inhibitor and topped up with DEPC water. The reaction was carried out at 37°C for 2h and stopped by adding 2µl of 200mM EDTA, pH 8. The RNA was precipitated with 2.5µl of 4M LiCl and 75µl of 100% ethanol for 1h at -20°C. After centrifugation step at 10000g for 15min, the pellet was washed once with 70% ethanol, air-dried and dissolved in 50µl of DEPC treated water.

2.2.4 Northern blotting and hybridisation

2.2.4.1 Blotting

After electrophoresis, the gel was washed briefly three times to remove the formaldehyde. In the blotting tank, 20x SSC (3M sodium chloride; 0.3M sodium citrate pH 7.5) was added for the transfer. A large Whatman paper was prewet with 20x SSC. This large Whatman had the ends within the transfer solution with two

smaller prewet Whatman pieces on top of this. The gel was placed with the wells facing the Whatman papers. The Hybond N nylon membrane was prewet in 20x SSC the membrane placed on top of the back part of the gel, with no air bubbles between the membrane and gel. Three more pieces of Whatman paper was added on top of the membrane, these prewet in 2x SSC. A stack of paper towels was added on top, and finally, a flat lid with a 500g weight. The transfer was carried out overnight at room temperature. At the end of the transfer, the gel and membrane were removed together, and the positions of the wells marked out. The membrane was washed one minute in 6x SSC and air dried between two Whatman papers and the RNA subsequently fixed on the membrane by baking the membrane for 30min at 120^{o}C. The RNA ladder was stained with 0.2% methylene blue/0.5M sodium acetate pH 5.2, and the bands differentiated with 20% ethanol.

2.2.4.2 Northern hybridisation

The membrane was washed briefly in 5x SSPE (20x SSPE: 3M sodium chloride; 0.2M sodium phosphate NaH_2PO_4; 0.02M EDTA; pH 7.4). Herring sperm heated at 95^{o}C, 10 minutes and then placed on ice, was added to a final concentration of 0.04 mg/ml to the prehybridisation solution (5x SSPE, 1x Denhardts [50x Denhardts:1% BSA, 1% Ficoll 400; 1% polyvinylpyrrolidon], 0.7% SDS, 50% Formamide). The membrane was prehybridised at 54^{o}C for two hours. Herring sperm was added again to a final concentration of 0.04 mg/ml together with the DIG-labelled probe to a concentration of 100ng/ml hybridisation buffer. The hybridisation was in the ratio 4:1 (Prehybridisation buffer:50% dextran sulphate). The herring sperm and RNA probe was heated at 95^{o}C, 10min before added to the hybridisation buffer. The membrane was hybridised overnight at 54^{o}C. The first set of stringency washes (2x SSC, 0.1% SDS) was at room temperature, 10 minutes, twice. The second set of stringency washes was at 65^{o}C, 20min, twice. The membrane was washed one minute at room temperature with PBT (1x PBS [10x PBS: 0.15M NaCl, 0.1M $Na_2HPO_4.2H_2O$, 0.1M $NaH_2PO_4.H_2O$]; 0.1% BSA; 0.1% Triton X-100), then blocked with PBT at room temperature for 30min. AP-anti-DIG antibody was added to a final concentration of 1:4000 to PBT. The membrane was then washed three times, 10min in PBT, then equilibrated one time, five minutes in AP buffer (sterile filtered, 0.1M Tris pH 9.5; 0.1M sodium chloride; 0.05M magnesium chloride). The substrates NBT

(nitrobluetetrazolium in 70% dimethylformamide) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) were added to the AP buffer to a final concentration of 0.225 mg/ml and 0.175 mg/ml respectively and membrane developed in the dark at room temperature. When bands appeared, the reaction was stopped by washing the membrane with water.

2.2.4.3 **In situ *hybridisation***

All steps were carried at room temperature unless otherwise stated. The animals were relaxed in 4% magnesium chloride in seawater for 30min then fixed in PFA/HEPES (0.1M Hepes; 0.42M sodium chloride; 2mM magnesium chloride; 4% paraformaldehyde), at room temperature for one hour. The samples were washed three times, 10min in PBST (1x PBS with 0.1% Tween). The samples were heated for 10min, 90^{o}C, then snap cooled on ice. The samples underwent Proteinase K (20 µg/ml PBST) treatment for 15min and the reaction stopped with glycine (0.1g/ml PBST), 5 minutes. The samples were washed twice again, 5min in PBST and then refixed in 4% PFA/PBS for at least 20min. The samples were then washed three times, 5min in PBST. Blocking was carried out with 0.5mg/ml PBST herring sperm, twice for 5min. The samples were then washed 5min in hybridisation buffer (50% Formamide, 5x SSC, 1mg/ml herring sperm, 0.1mg/ml heparin, 0.1% Tween 20) and prehybridised at 50^{o}C for two hours. The DIG-labelled RNA probe was added to an end concentration of 100ng/ml hybridisation buffer and hybridised at 50^{o}C, overnight. The probe was heated at 70^{o}C, 10min and cooled on ice before addition to the hybridisation buffer.

The first stringency wash (50% Formamide, 2x SSC, 0.1% Tween 20) was at 50^{o}C, one hour. The second stringency wash (2x SSC, 0.1% Tween 20) was at 50^{o}C for 15min and the last stringency washes (0.2x SSC, 0.1% Tween 20) were at 50^{o}C, 15min, twice. These were then washed briefly for 5min with PBST. Blocking of samples was carried out for one hour with 1% BSA/PBST before incubation at 4^{o}C, with anti-DIG-alkaline-phosphatase conjugated antibodies, added to an end dilution of 1:10000 in 1% BSA/PBST. After the antibody binding, the samples were washed in PBST, four times, 20min each. The samples were equilibrated, three times for 5min in AP buffer, and NBT and BCIP was added to AP buffer to a final concentration of 0.225 mg/ml and 0.175 mg/ml respectively and the samples were allowed to develop in the dark. The substrate reaction was stopped with Stop Mix (10mM EDTA in

PBST) by washing three times for 5min. The samples were then mounted in mounting medium (Glycerol:Stop Mix, 9:1)

2.2.5 Reverse transcription (RT)

Reverse transcription was carried out with the kit RevertAid H minus M-MuLV reverse transcriptase using the standard protocol of MBI Fermentas. 3µg of RNA were dissolved in 5µl of DEPC-water heated 5 min at 70°C and added to the following mixture:

Component	Amount	Final
5X RT buffer (50Mm Tris pH 8.3, 50mM KCl, 4mM $MgCl_2$)	4µl	1x
dNTPmix (10mM)	2µl	1mM each
oligodT primer (10 µM)	2µl	1µM
Reverse transcriptase (200U/µl)	1µl	10U/µl
DEPC-water	6µl	

The cDNA synthesis was performed at 42°C for 60min, and the sample was finally heated at 70°C for 10min to stop the reaction.

2.2.6 Rapid Amplification of cDNA ends (RACE)

The RACE approach is a specially designed RT-PCR for generating full-length cDNAs. The RACE was performed according to the *SMART*^{tm} (Clontech Laboratories, Inc) protocol. For 5' RACE-PCR, the FzR primers were used and for the 3' RACE-PCR, the FzF primer (see the primer list) for the specific primers. The cDNA clone was obtained by overlapping fragments amplifie by RACE-PCR.

2.2.6.1 First strand cDNA synthesis:

Component	Amount	Final concentration
RNA sample (total RNA)	1µg	
SMART II oligo (20µM)	1µl	2µM

5’ or 3’ CDS primer (20µM)	1µl	2µM

The mixture (end volume 10µl) was heated to 70°C for 2min, chilled on ice for 2 min, briefly centrifuged and the following components were added:

Component	Amount	Final concentration
5x first strand buffer	2 µl	1x
DTT (100mM)	1µl	10mM
dNTP mix (10mM)	1µl	1mM
PowerScript™ Reverse Transcriptase	1µl	

The samples were incubated at 42°C for 90min, diluted with 100µl Tricine-EDTA buffer (10mM Tricine-KOH (pH 8.5), 1mM EDTA), heated at 72°C for 7 min, and then stored at -20°C.

2.3 Methods related to DNA

2.3.1 Genomic DNA Isolation

Tissue material approximately the size of rice grain was collected, and 750µl of CTAB buffer (2x CTAB, 2x SDS, 0.1M Tris pH 8.0, 1.4M NaCl, 0.02M EDTA, pH 8.0) and Proteinase K solution (to an end concentration of 0.2 mg/ml) added to it. The tissue was macerated with a sterile pin, incubated at 65^{o}C for 2h with intermittent light vortexing. Another lot of Proteinase K solution was added and incubated overnight. The next day, 1V phenol:chloroform:isoamylalcohol (PCI) in the ratio (25:24:1) was added to the mixture, and the contents mixed by inverting. It was then spun at 10000 rpm for 10min. The top aqueous layer was collected and the PCI step repeated if flocculent matter were in the aqueous layer. 1V CI (24:1) was added to the aqueous mixture and mixed by inverting. Samples were spun at 12000 rpm for 12min. The supernatant was collected and 2-2.5V ice-cold 95% ethanol and 10% v/v sodium acetate was added and the solutions mixed by inverting. The precipitation was carried out at –80^{o}C for 20min, or 20^{o}C, overnight. The precipitate was obtained by spinning at 12000 rpm for 15-20min at 4^{o}C. The pellet was washed twice with 750-1000µl,

70% ice-cold ethanol, spun at 5000 rpm for 5 minutes at 4°C. The genomic DNA was resuspended in 35µl DEPC-treated water, and stored at –80°C.

2.3.2 Standard RACE-PCR protocol

Component	Amount	Final concentration
10X PCR buffer with $(NH_4)_2 SO_4$	2.5µl	1x
$MgCl_2$ (25mM)	4.0µl	4mM
dNTP mix (10mM)	0.5µl	0.2mM
antisense primer (5'RACE) or sense primer (3'RACE) (10µM)	1.0µl	0.4µM
10xUPM primer mix (0.4µM UPM short, 2µM UPM long)	1.0µl	1x
3' or 5' cDNA (RT reaction)	2.0µl	≈ 10ng
Taq polymerase (1U/µl)	1.0µl	1U/25µl
water	up to 25.0µl	

The reaction was performed in the Biometra thermocycler with the following cycle program:

Denaturation	94°C → 2 min
30 cycles	
Denaturation	94°C → 40 sec
Annealing∗	52-64°C → 40 sec
Extension	72°C → 3 min 30 sec
Final extension	72°C → 10 min

Taq polymerase (1-2U) was added during the first denaturation step (*Hotstart*).

∗Depending on the specific primer melting temperature.

2.3.3 T/A cloning

The Taq-polymerases without proofreading activity have the ability to add additional deoxyadenines to the 3' end of a double-stranded DNA fragment. PCR fragments with A-overhangs can be easily cloned into linearised vectors with T-overhangs. Such vectors are commercially available (e.g. pGEM-T, Promega), or made by adding T-overhangs to the linearized vector (e.g. Bluescript) using Taq polymerase.

2.3.3.1 T-overhang Bluescript vector

Bluescript vector (~14µg) was digested with 20U EcoRV restriction enzyme (final volume 60µl), incubated for 2h at 37°C. The digestion product, the uncut vector as control and the band of linearised vector were loaded on a 1% agarose gel. Bands were excised and gel eluted using the *NucleoSpin® Extract* kit. 1-2µg of purified vector was used in the following T-tailing reaction.

Component	Amount	Final concentration
Linearized Bluescript	1-2µg	
Taq DNA polymerase (5U/µl)	1µl	0.5U/µl
dTTP (2mM)	1µl	0.2mM
10x PCR buffer	1µl	1x
$MgCl_2$ (25mM)	0.6µl	1.5mM
Water	up to 10µl	

The reaction was incubated at 70°C for 30min. Reaction was purified using *NucleoSpin® Extract*.

2.3.4 Ligation into pGEMT vector

Ligation was carried out according to the manufacturer's (Promega) protocol. 3µl of PCR product was added to a mixture containing 5µl ligation buffer, 1µl vector and 1µl T4 Ligase. The reaction was carried out at 4°C, overnight.

2.3.5 Ligation into generated pBluescript T-overhang vector

The amount of vector and insert was variable in order to achieve 1:1 to 1:3 vector/insert ratio. 1µl of 10x ligation buffer and 1U of T4 DNA ligase were added, and the final volume was adjusted to 10µl with double distilled (dd) water. The reaction was carried out at 4°C, overnight.

2.3.6 Transformation

Heat shock competent cells

E.coli XL-1 Blue strain was used for transformation. 2ml of overnight culture were transferred into 50ml of LB medium, and incubated at 37°C until the cells reached a logarithmic phase OD_{600nm}= 0.4. They were then incubated on ice for 15min and centrifuged at 6000g, 10min at 4°C. The bacterial pellet was resuspended in 80ml TFB1 (10mM MES, 100mM RbCl, 10mM $CaCl_2 * 6H_2O$, 50mM $MnCl_2 * 4H_2O$, pH5.8), incubated on ice for another 15min, and again centrifuged. The bacterial pellet was resuspended in 8ml TBF2 (10mM MOPS, 75mM $CaCl_2 * 6H_2O$, 10mM RbCl, 15% glycerol, pH 6.5). The competent cells were divided in 100µl aliquots, frozen in liquid nitrogen and stored at -80°C.

2.3.6.1 Heat shock transformation

Frozen competent cells (100µl) were thawed on ice for 10min. After addition of 1-5µl of ligation reaction (0.1-0.5 pmol of plasmid) and the cells were incubated on ice for 30min. They were then heated at 42°C for 3min and then chilled on ice for 2min. 1ml of TY (2% Tryptone, 0.05% yeast extract, 0.0058% NaCl, 20mM $MgS0_4 * 7H2O$, pH 7.0) medium was added to it and the cells were further incubated at 37°C for 1h, shaking at approximately 220 rpm. 50-200µl of the cell suspension was then plated out onto LB-Carbenicillin agar plates. For white/blue selection of bacterial clones containing vector with an insert, X-Gal (40µl of 3% solution in DMSO) and IPTG (4µl of 100mM solution in water) were plated on a LB-Carbenicillin agar plate prior to transformation. Plates were left to dry at room temperature until the liquid had adsorbed to the surface. Plates were then incubated overnight at 37°C.

2.3.6.2 Electrocompetent cells

For preparation of electrocompetent cells (*E.coli* XL-1 Blue strain), 2ml of overnight bacterial culture were transferred into 500ml of LB medium and incubated at 37°C until they reached an of OD_{600nm}= 0.4. They were then incubated on ice for 15min and centrifuged at 4000g for 10 min at 4°C. The bacterial pellet was then washed with 500ml of ice-cold MiliQ water and again centrifuged. The pellet was resuspended in 2ml GYT-medium (10% glycerol, 0.125% yeast extract, 0.25% tryptone and then cells were diluted 1:100mM MES, and OD_{600nm} was determined. The concentration of the cells should be 2-3 x 10^{10} cells/ml (1 OD_{600nm} = 2.5x10^8 cells/ml). The conductivity of the suspension was measured in an electroporation cuvette. If the conductivity was more than 5mEq, the cells were further diluted. The cells were then divided in 40µl aliquots, frozen in liquid nitrogen and stored at -80°C.

2.3.6.3 Electroporation

Aliquots of electrocompetent cells (40µl) were thawed on ice and 1µl of the ligation product was added to the cells, mixed and transferred into a cooled electroporation cuvette (Bridge, Spaltweite 2.5mm). The electroporation was performed in *EQUIBIO Easyject Plus Pulser* with parameters: 2.5 kV, 25µF, 201 Ohm for 5ms. After electric pulsing, the bacteria were immediately mixed with 1ml TY-medium and shaken at 37°C for one hour. 50-200µl of the cell suspension was then plated out onto LB-Carbenicilin agar plates and incubated overnight at 37°C.

2.3.7 Bacterial culture

2.3.7.1 On agar plates and blue/white selection

The bacteria were cultured on agar plates (1% tryptone, 0.05% yeast extract, 0.085M NaCl, pH 7.5, 1.5% agar) at 37°C. In order to of transformed bacteria for antibiotic resistance, carbenicillin (final concentration of 100µg/µl) was added to the medium before pouring the plates. For white/blue selection of bacterial clones containing vector with an insert, X-Gal (40µl of 3% solution in

DMSO) and IPTG (4µl of 100mM solution in water) were plated on a plate 1h prior to plating the bacteria.

2.3.7.2 Liquid bacterial culture

The bacteria were cultured in LB-medium (1% Tryptone, 0.05% yeast extract, 0.085M NaCl, pH 7.5) at 37°C in a shaker. An overnight culture contained 1-2x10^9 cells/ml. The bacterial titer was determined photometrically (1 OD_{600nm} = 2.5x10^8 cells/ml). Transformed bacteria were cultured under selective pressure of carbenicillin, at a final concentration of 100µg/µl. For a long-term storage (several years) an overnight culture, the bacterial culture was mixed with glycerol (20% final concentration), and stored at -80°C.

2.3.8 Plasmid purification

To isolate plasmids from bacteria, up to 15ug of DNA was used for a *miniprep* using the *NucleoSpin® Plasmid Purification Kit* and for preparation of up to 100µg DNA, the (*midi prep*) *Qiagen® Plasmid Midi Kit* were used, both according to the protocols of manufacturers *Macherey & Nagel* and *Qiagen.*

2.3.9 Plasmid restriction analysis

In order to check the size of the insert, the plasmid vectors were digested with different restriction enzymes. Restriction enzymes were chosen according to the recommendation of the manufacturer (MBI Fermentas). The amount of enzyme per reaction was calculated according to the unit definition (1U of enzyme hydrolyses 1µg of DNA per hour). Analytical digestions contained 0.5-1µg DNA, and for preparative purposes 1-10µg of DNA were used. Digestion with two enzymes was performed when possible in *Universal Y=/Tango™ Buffer* system according to the manufacturer's recommendation. For incompatible enzymes, digestion was performed first with one enzyme and after its deactivation at 80°C and phenol chloroform purification of DNA, the second digestion was carried out.

2.3.10 DNA sequencing

DNA sequencing was performed using the commercial services of two sequencing companies *(Seq Lab* and *Medigenomix*). The sequencing reaction was carried out with either standard or specific primer, and the results were provided as a text file and electropherogram that enabled manual editing of sequencing data.

2.3.11 Establishment of microsatellite library

2.3.11.1 Digestion with 4-base cutter and size selection

Genomic DNA was isolated as described in Section 2.3.1. Using 10-20µg genomic DNA, a 4-base cutter, e.g. Mbo1 (2-3U) was used to digest the reaction overnight, at 37°C. The reaction was ran on 1% agarose. A DNA smear containing small to large fragments denoted completion of the digestion. The remaining reaction mixture was ran on a 0.8 % agarose gel. The gel band between the size range 200-700 bp was excised from the gel and the DNA purified using *NucleoSpin® Extract*.

2.3.11.2 Annealing of linkers and ligation to DNA

SAULA and SAULB were added together, heated at 70°C, 10 min and left for 1h at room temperature. For every 200ng of digested DNA, 2µg of annealed linkers were added, 2-4U T4 ligase, 1X ligation buffer. The ligation reaction was incubated at 16°C, overnight.

2.3.11.3 Amplification of ligated products

Component	Amount	Final concentration
Ligation mixture	2.0µl	
10X PCR buffer with $(NH_4)_2 SO_4$	2.5µl	1x
$MgCl_2$ (25mM)	1.5µl	1.5mM
dNTP mix (10mM)	0.8µl	0.32mM
SAULA (10µM)	2.5µl	1.0µM
Taq polymerase (1U/µl)	2.0µl	2U/25µl
water	up to 25.0µl	

Only 5' phosphates are available on digested genomic fragments. After the ligation step, only the 3' end of SAULA of the double stranded linker is covalently bound to the Mbo1-digested fragment and the other is held on by complementary base pairing. Hence the first step of the PCR involved an extension step that enabled Taq polymerase to heal the nick between genomic DNA and the linker sequence.

Heal nick	72°C → 5 min
30 cycles	
Denaturation	94°C → 30 sec
Annealing	67°C → 30 sec
Extension	72°C → 90 sec
Final extension	72°C → 10 min

An aliquot was ran on a 2% agarose gel check for the correct amplification in the correct size range.

2.3.11.4 First capture of CA-repeats with 5'-biotinylated probes

In order to hybridise the DNA to the 5'-biotinylated probes, one round of cycling (94°C → 5 min, 55°C → 1 min, 72°C → 10 min) was carried out on the following mixture:

Component	Amount	Final concentration
SAULA PCR mixture	3.0µl	~300ng
10X PCR buffer with $(NH_4)_2$ SO_4	2.0µl	1x
$MgCl_2$ (25mM)	1.6µl	2.0mM
dNTP mix (10mM)	1.6µl	0.80mM
Biotin-CA (1µM)	2.0µl	0.1µM
Taq polymerase (1U/µl)	0.5µl	0.5U/20µl
water	up to 20.0µl	

This PCR reaction was subsequently purified using *NucleoSpin® Extract*.

2.3.11.5 Second capture, of target molecule-genomic hybrids

The streptavidin MagneSphere® paramagnetic particles (Promega) were washed according to manufacturer's instructions. Purified products were added with 120µl of 6x SSC/0.1% SDS to the particles, mixed and incubated at room temperature for 15min. After each capture with the separation stand, the particles were washed with 150µl of 6x SSC/0.1% SDS, for 15min at the following temperatures: 60°C, 65°C, 70°C, 75°C. Final two washes were carried out with 6x SSC at 80°C, 15min. DNA was eluted with 100µl, 0.1M NaOH at 80°C, 10min and subsequently neutralised with 100µl TE, pH 7.5.

2.3.11.6 Amplification of captured target CA-repeat DNA

Component	Amount	Final concentration
Enriched mixture	2.0µl	
10X PCR buffer with $(NH_4)_2 SO_4$	2.5µl	1x
$MgCl_2$ (25mM)	1.5µl	1.5mM
dNTP mix (10mM)	0.8µl	0.32mM
SAULA (10µM)	2.5µl	1.0µM
Taq polymerase (1U/µl)	2.0µl	2U/25µl
water	up to 25.0µl	

Denaturation	94°C → 5 min
30 cycles	
Denaturation	94°C → 30 sec
Annealing	67°C → 30 sec
Extension	72°C → 90 sec
Final extension	72°C → 10 min

The PCR products were assessed on a 2% agarose gel. Another round of enrichment (first and second capture, PCR of captured products) was carried out.

2.3.11.7 Cloning of enriched fragments into pBluescript SK+ vector

The PCR products were digested overnight at 37°C to remove linkers as follows:

Component	Amount	Final concentration
Enriched PCR mixture	5.0µl	~500ng
10x Buffer	2.5µl	1x
Mbo1 (10U/µl)	1.0µl	1U
water	up to 25.0µl	

The vector was digested similarly, but the restriction enzyme BamHI was used instead. The cut vector was then dephosphorylated (1U CIAP to 1µmole vector/minute) to prevent self-ligation at 37°C, 20min, then inactivated at 55°C for 20 min, followed by 75°C for 10 min. The dephosphorylated vector was then purified with the *NucleoSpin® Extract*. Ligation of the cleaned dephosphorylated vector and digested enriched inserts was carried out at a ratio of 1:2, for 2 h at 22°C, and the reaction stopped by heating contents at 65°C for 10min. The ligation mixture was transformed as described in Section 2.3.6.

2.3.11.8 Colony hybridisation

The nylon membranes (Nytran, Schleicher & Schuell) were placed on agar plates for 5 min. A sterile needle made asymmetrical pattern on the membrane and the patterning marked out on the underside of the Petri dish for correct orientation later when identifying positive colonies. After removal, the membranes were placed (colony side up) onto Whatman papers soaked in solution 1 (0.5M NaOH, 1.5M NaCl) for bacterial lysis and DNA denaturation for 10 min. Following the same procedure, membranes were neutralised with solution 2 (0.5M Tris-HCl, pH 7.5, 1.5M NaOH) 10min, then incubated with 2x SSC for 5min. Membranes were left to dry at room temperature and DNA fixed in an oven at 80°C, 1h.

2.3.11.9 Hybridisations to detect CA-repeats

The membranes were deproteinated at 45°C for 50min in solution R1 (2X SSC, 0.1% SDS) with 0.1 mg/ml Proteinase K. Two more incubations were made with solution R2 (1X SSC, 0.1% SDS) at 45°C for 10min. The membranes were then prehybridised at 45°C for 2h, in hybridisation buffer (5X SSC, 1% BSA, 0.02% SDS, 0.1% N-lauroylsarcosine), and hybridised overnight at 45°C, with 0.3pmol/ml buffer of the DIG-$(CA)_{12}$ oligonucleotide probe.

2.3.11.10 Stringency washes and detection

After hybridisation, the membranes were washed twice at 45°C for 12min in solution R1, then twice at 45°C for 10min in solution R2. The membranes were then equilibrated with buffer B1 (0.1M maleic acid, 0.15M NaCl, 0.2M NaOH, pH 7.5) twice at 45°C for 5min. The membranes were then blocked with buffer B2 (1% BSA in Buffer B1) at 45°C for 40min. All subsequent steps were carried out at room temperature. The membranes were then incubated 30min, with Anti-DIG-AP-conjugate/Buffer 2, in a ratio of 1:2500. The membranes were then washed twice at 12min with Buffer B2 and then equilibrated in detection buffer B3 (0.1M Tris-HCl, pH 9.5, 0.1M NaCl, 50 mM $MgCl_2$) for 5min. The substrates NBT and BCIP were added to the buffer B3 to a final concentration of 0.3375 mg/ml and 0.175 mg/ml respectively and the membranes developed in the dark. When the colour had developed sufficiently, the reaction was stopped by rinsing the membranes with water and dried at room temperature.

After allocating the correct patterning of membranes to the bacteria plates, corresponding positive clones were picked and grown in liquid culture and stored as described in Section 2.3.7 and the plasmid purified as in Section 2.3.8. A second round of detection for positive clones were carried using dot blots of the plasmid DNA and the hybridisations carried out as above. After restriction digestion with BamHI as described in Section 2.3.9, confirmed positive clones with appropriate size lengths were sent for sequencing as described in Section 2.3.10.

2.3.12 PCR conditions for amplifying microsatellites

Component	Amount	Final concentration
Genomic DNA	2.0µl	~200ng
10X PCR buffer with $(NH_4)_2 SO_4$	2.5µl	1x
$MgCl_2$ (25mM)	1.5µl	1.5mM
dNTP mix (10mM)	1.0µl	0.4mM
Forward primer (10µM)	1.0µl	0.4µM
Reverse primer (10µM)	1.0µl	0.4µM
Taq polymerase (1U/µl)	1.0µl	1U/25µl
water	up to 25.0µl	

Denaturation	94°C → 5 min
30 cycles	
Denaturation	94°C → 30 sec
Annealing∗	50-60°C → 30 sec
Extension	72°C → 30 sec
Final extension	72°C → 5 min

Taq polymerase (1-2U) was added during the first denaturation step (*Hotstart*).

∗Depending on the specific primer melting temperature.

2.3.13 Checking for locus polymorphism with polyacrylamide gels (PAGE)

2.3.13.1 Treatment of glass plates and gel preparation

The glass plates were washed with soap to ensure that the glass surfaces were without any grease. They were dried with Kimwipes and the full Glass Plate was then treated with Bind-Silane for at least one hour, the Bind-Silane rinsed off with MilliQ water and ethanol and subsequently dried again with Kimiwipe. The smaller glass plate was treated with BlueSlick, rinsed with MilliQ water, as previously described. To ensure that the wells formed later were of a tight fit, the spacers and comb picked had to be of the same width (otherwise the comb width should be larger than the spacers). The treated sides of glass were placed towards each other. The sides were sticky taped, ensuring the absence of air bubbles. The bottom and lower sides of glass

were reinforced with more sticky tape. A spout was made on one side of glass plate for the gel solution when pouring. Tilting the glass plate at an angle, the gel solution was poured in a gradual flow to ensure no formation of bubbles and the plates tilted backwards for upward movement of the air bubbles when these formed. The shark-tooth comb was placed at the top, with straight side down, to create a space for the wells. This spacing was not too deep, to ensure that the teeth from the comb will reach this front later in order to create wells. The remaining gel solution was poured onto the comb area. From the comb area, the glass plates were tilted slightly upward. If the gel was not used after polymerisation, exposed area was covered with wet towels and kept at 4^{o}C.

2.3.13.2 Running of samples on 8% PAGE

A 8% PAGE (30 ml 19:1 Bis:Acrylamide, 1x TBE, 7-8M urea) was made with slight warming to dissolve the urea. Solution was filtered and 75µl TEMED and 1.5 ml, 10% APS added for polymerisation and the gel was cast. After polymerisation, the gel was placed in running buffer (1x TBE, fresh) and warmed by prerunning the gel at 60W (this must kept constant to ensure continual correct heating) and 1800V (maximum) for at least one hour. Integrity of the wells were checked by adding loading buffer (8:1, Formamide:6x loading buffer). DNA samples and marker were prepared in the ratio 1:1 with loading buffer. The samples were heated at 80^{o}C for 3min to denature the DNA. The samples were then put immediately on ice, quickly spun down before loading. Samples were loaded and the gel ran at 40W (this must kept constant to ensure continual correct heating) and 1800V (maximum).

2.3.13.3 Silver Staining

Gels were fixed in 10% acetic acid, for 40min. This solution was kept aside for the stop reaction. The gel was washed twice, 20min, in dd water to remove the acid. A second fixing step was carried out with 1% nitric acid for 1min and washed twice in dd water for 1min. Silvering was carried out for at least 1h with 0.1% silver nitrate, 0.037% formaldehyde. The gel was rinsed briefly and then development was carried out with pre-cooled developing solution (0.24M sodium carbonate, 0.037% formaldehyde, 0.002% sodium thiosulphate). Developing was stopped with 10%

acetic acid, 5 min. The gel was washed twice with dd water and impregnated in glycerol solution (70g glycerol:500 ml water).

2.4 Histochemistry and immunostaining

2.4.1 Detection of I-cells, nematoblasts and nematocytes

For cytological examinations, primary polyps and colonies grown on glass slides were fixed with Lavdovsky's fixative (formaldehyde 5 ml + acetic acid 2 ml + EtOH 25 ml + water 20 ml) overnight at 6^{o}C. Samples were permeabilised with Sörensen's buffer, pH 7.0 supplemented with 1% Triton-X100, 1h, and subsequently stained with May-Grünwald (3.5h), followed by Giemsa solution (3.5h). Intermediate washing between stainings were carried at with Sörensen's buffer, 1h. Final destaining was carried out with Sörensen's buffer.

2.4.2 BrdU labelling and RF-amide labelling

Animals were incubated 1-2h in 0.02 mM BrdU solution in seawater. Animals were subsequently fixed with 4% paraformaldehyde overnight or at 4^{o}C for at least 1h at room temperature. All subsequent steps were carried out at room temperature. Samples were permeabilised with wash 1 (0.1M Na_2HPO_4, pH 7.0) for 1h, then neutralised with 0.4M glycine for 2h. Denaturation of DNA was carried out with 2N HCl, 1 h. Prior to antibody incubation, samples were blocked in 0.25% Triton-PBS. Anti-BrdU antibody (Roche) incubation was carried at 4^{o}C, overnight, at ratio of 1:500 in 0.25% BSA in 0.25% Triton-PBS. Samples were washed in 0.25% Triton-PBS, 1 h, room temperature and the secondary antibody anti-mouse-IgG-AP (Roche) added to 0.25% BSA in 0.25% Triton-PBS I the ratio 1:500. Samples were incubated at 4^{o}C, overnight. Samples were washed 3X, 10 min in 0.25% Triton-PBS. Samples were equilibrated three times for 5min in AP buffer (see Section 2.2.4). NBT and BCIP were added to the AP buffer to a final concentration of 0.225 mg/ml and 0.175 mg/ml respectively and the samples allowed to develop in substrate reaction in the dark. The reaction was stopped with stop mix (See Section 2.2.4), three times, each lasting 5min.

For detection of nerve cells, animals were fixed with 4% paraformaldehyde, then washed with 0.4M glycine+1%Tween, pH 7.0, for 4h, further permeabilised with 0.25% Triton-PBS for 1-2h. Samples were then blocked with PBS containing 5%

BSA for 1 h, and incubated for 4h at room temperature with the primary antibody (gift from Cornelius Grimmelikhuijzen). Samples were washed twice, 30min with 0.25% Triton-PBS, Incubation with secondary antibody, anti-rabbit IgG labelled with Alexafluor 488 (Molecular Probes) was carried out in the ratio 1:80 in with 0.25% Triton-PBS, 3h. Samples were washed twice, 15min with 0.25% Triton-PBS. Samples were counterstained with Evans's Blue (0.1% in PBS), 1min and destained with 0.25% Triton-PBS until no stain leaked from the samples.

2.5 Raising and culturing of animals

Hydractinia colonies were cultured in artificial seawater as described in Frank et al., 2001. Colonies could be subcloned via explants when necessary. A life cycle of *Hydractinia* from Müller and Leitz (2002) is shown in Figure 4. At the planula larvae stage, the animals could be artificially induced to metamorphosis with 58 mM cesium chloride ions by stimulating neurosensory cells with depolarisation (Müller and Leitz 2002). Hence all stages from *Hydractinia* from embryogenesis to post-induction (PI) could be used for experimental purposes.

2.6 Treatment with GSK3-inhibitors: azakenpaullone and alsterpaullone

5 mM stock solutions of alsterpaullone (9-nitro-7,12-dihydroindolo[3,2-d][1]benzazepin-6(5H)-one) (Carbiolchem) were prepared in DMSO and further diluted to a concentration of 0.2-0.1 mM/L in DMSO. When treating animals with alsterpaullone, 1 µl of this DMSO stock solution was added to the seawater to obtain a final solution containing 0.2-0.01 µM alsterpaullone in 1 ppm DMSO. Control animals were treated with 1 ppm DMSO. Azakenpaullone (Kunick et al., 2004) was a gift from Dr Conrad Kunick, Technical University of Braunschweig, Germany and was handled similarly to alsterpaullone. Animals with 3-day treatment regimes had the solution changed daily.

2.7 Statistical tests

All statistical tests were carried with free software available on the web. The Mann-Whitney U tests were carried out from: http://eatworms.swmed.edu/~leon/stats/utest.html and the chi-squared tests were carried from:http://schnoodles.com/cgi-bin/web_chi.cgi.

2.8 Fragment of *Hydractinia vasa*

The plasmid obtained for the transcription of the RNA probe was a gift from Dr Nicole Rebscher, Inst. Of Zoology, University of Heidelberg, Germany. The span of sequence used is 251 base pairs long, and a BLASTx search of this sequence gave a high homology to the Vasa-related protein CnVAS1 [Hydractinia echinata], accession number: BAB13686.

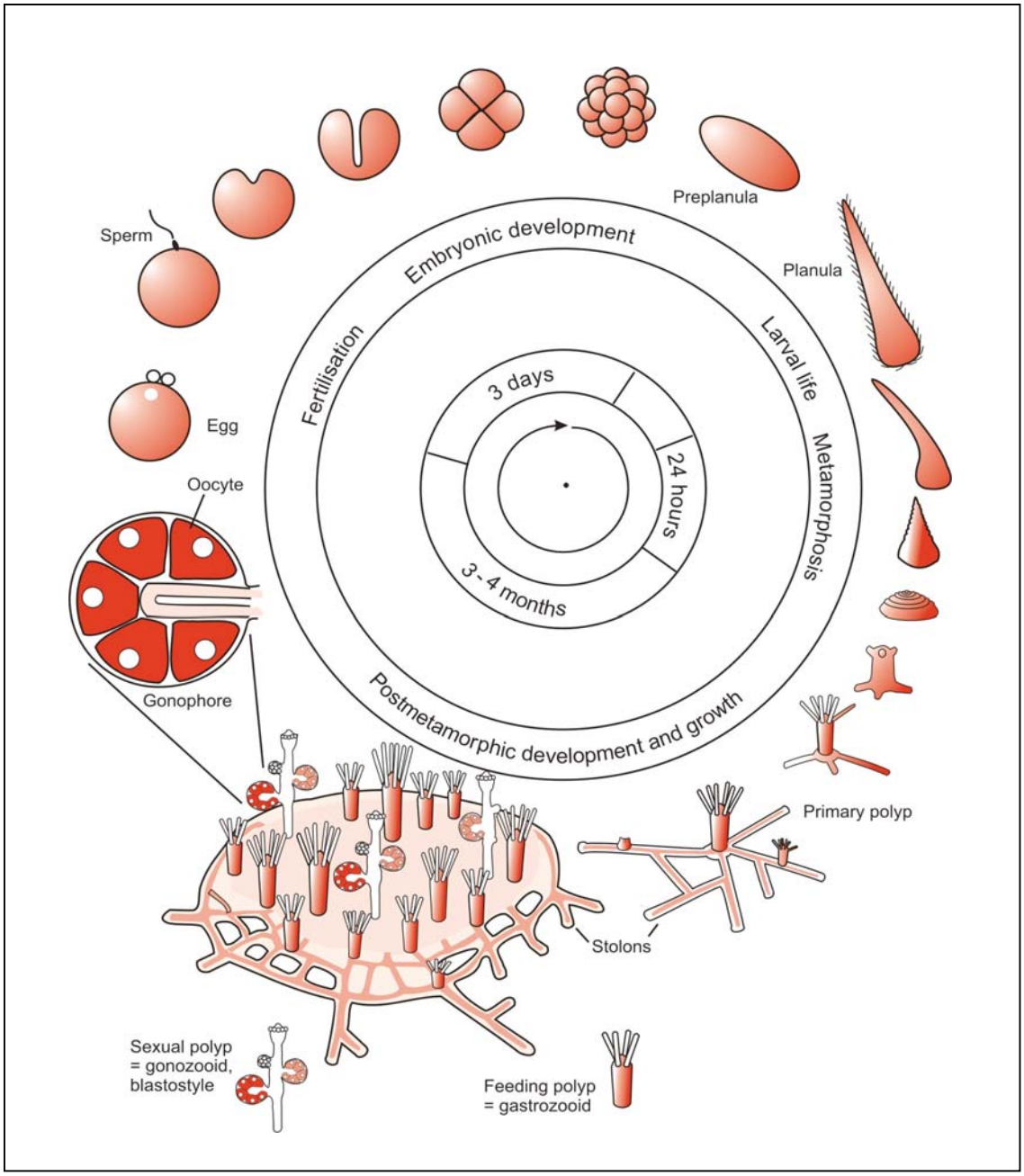

Figure 4. Life cycle of *Hydractnia* (Müller and Leitz 2002).

Results

3.1 *He-frizzled* is conserved evolutionarily and has motif required for canonical Wnt signalling

The cDNA clone obtained from 3' and 5' RACE has an open reading frame of 1680 bp, corresponding to 560 amino acids. A search through the NCBI Protein-protein BLAST gave a predicted primary structure of the protein that showed significant sequence similarity to Frizzled proteins of other organisms with closest similarity to *Hydra* followed by Frizzled-2 and Frizzled-7 of the other organisms. At the N-terminal, there is a cysteine-rich domain with ten cysteines (amino acids 38-147), typical for other known Frizzleds, and a prediction of transmembrane helices and topology of proteins (Tusándy and Simon 2001) confirmed the presence of seven putative α-helical hydrophobic domains known to Frizzleds (amino acids 223-519) (Fig. 5), resembling the family of G-protein-coupled receptors (GPCR) .The presence of the motif KTXXXW after the last transmembrane region, where the lysine, threonine and tryptophan positions represent conserved residues found to be essential in all Frizzleds for ß-catenin signalling (Umbhauer et al., 2000). The cloned cDNA-sequence will be subsequently termed *He-frizzled1*.

A ClustalW alignment (Appendix 2), of the putative protein sequence of *He-frizzled1* with other Frizzled sequences (Fig. 6), showed its highest relationship to that of *Hydra*, and this Hydrozoa group was more closely related to the vertebrate Frizzled-2, -7, and –10, despite the presence of the protochordate *Ciona*, the invertebrate *Drosophila* and the sponge *Suberites*.

MEIIFTKLFF FVIVLLATVN LSHAGQKTTC VSLKHRGLRL CQNLGYNATM FPNSLEHRNM

ENASTELDNF LPLVKIRCAK EIEFFLCSVY LPVCLDSGPL PPCREVCERA QRGCIKLMTQ

YGFPWPEYLR CSRFPKKVED RLCVDKPFKE PGTNGNNGNN GINTGGIPVN PSHNTTHATY

TM1

DDYRCPAKQQ KETKHYKFMG TEKCASLCTP IYFTHKEKDF ARNWVLFWSV VCMLSTAFTL

TM2

LAFIVDMPRF RYPERPIIFL SGCYFIVAIA FISGPVSDNA IACHKTDDGT QLLNQGTDNA

TM3 TM4

SCTIVFMLIY FFLMSASIWW VILTLTWFLA AGLKWGHEAI EGSSQYFHAA AWAIPAAKTI

TM5

AVLAMNQIDG DILAGVCFVG GKNVNALRGF VLAPLFVYLV VGSFFLFAGF ISLIQIRKVL

TM6

KDDSSLRAEK LTRLMVRIGI FSILYSLPAI IVIACLFYEQ TYRIEWDSSW ISAWLTMLGK

TM7

CDEKVCFEFS GKSRPDFAVF MIKYLMLLMV GITSGFW IWS GKTIQSWRRF YYNKILHQRV

PRQYYESKDG SVFTARTNLT

Figure 5. Protein sequence of *He-frizzled* with putative Frizzled domains (red) and the seven transmembrane domains (blue). Motif KTXXXW (green) denote the putative highly conserved region found in all Frizzleds for activation of the Wnt/β-catenin pathway.

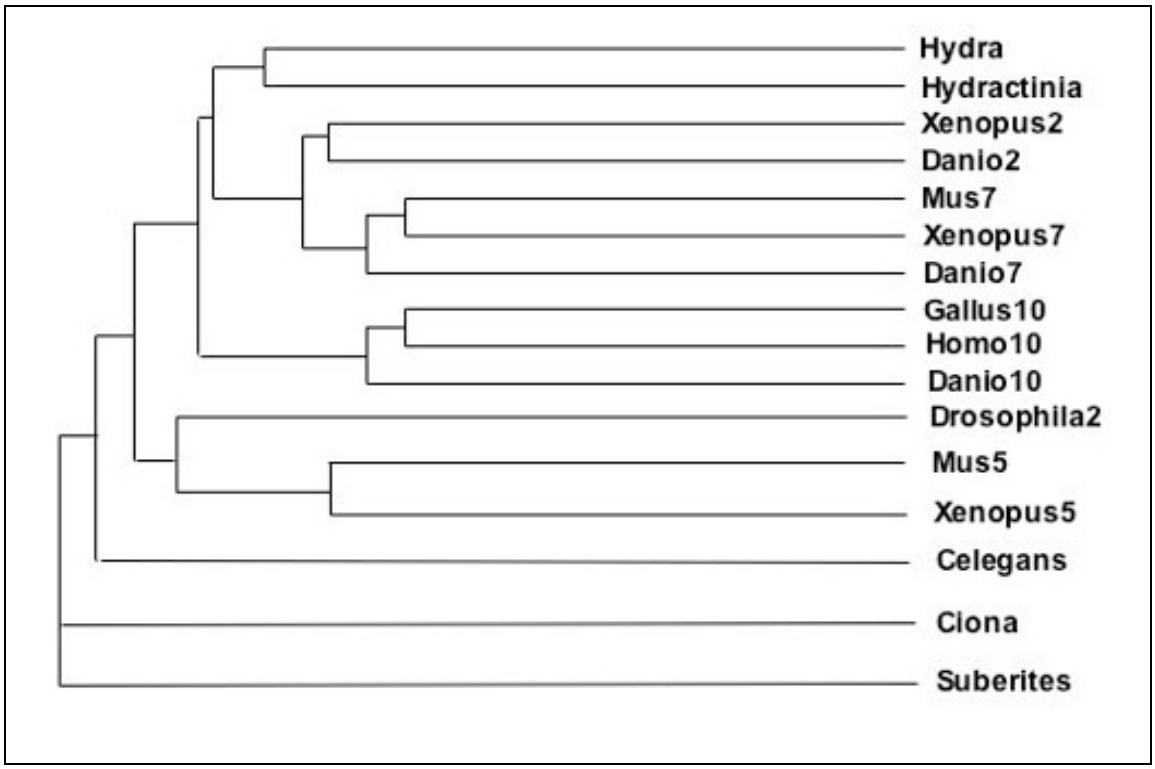

Figure 6. Cladogram of Frizzled protein relationships obtained from CLUSTALW alignment. Generic names given. Celegans = *Caenorabditis elegans*. Numbers indicate specific Frizzled homologues for that species. Numbers following the genus name denote Frizzled family. List of protein sequences and their accession numbers are given in the Appendix 1.

3.2 *He-frizzled* in embryonic and post metamorphic stages

3.2.1 Northern blot analysis

An RNA probe (amino acids 361-475) generated from the transmembrane region was tested for its expression in different developmental stages (Fig. 7) in a Northern analysis. A major transcript of approximately 2000 kb was expressed from egg throughout embryogenesis and development, until maturity. Another band at 800 bp was expressed intermittently, but highest during the mature stages. This band was first faintly observed during embryogenesis at preplaunula, a stage corresponding to the time point of gastrulation. Another band at 1800 kb was observed at approximately 28 hours after induction of metamorphosis. At this point of development, new structures essential to the developing primary polyp are being formed. All these bands could represent either isoforms or paralogues.

3.2.2 *In situ* hybridization*: Overview*

In situ analyses indicated that in the early stages of embryogenesis, the *He-frizzled* messag*e* was already present (Fig. 8A, C, E), ubiquitously distributed in the cytoplasm (Fig. 8F). After the onset of gastrulation (Figs. 8G-H), higher expression was observed in the central endodermal region of the preplanula and planula. This region coincides with the site where the first I-cells, nematoblasts and neuroblasts are known to arise (Van de Vyer 1964). At the posterior tip of the planula, where stinging cells reside, there is absence of expression.

After metamorphosis, there was general staining in cells undergoing proliferation. A broad circumferential band of stronger expression in the middle portion of the developing polyp at 18 hours post induction (Fig. 9B) was observed. This could be attributed to events that lead to the expansion of the gastric region. At 27 hours post metamorphic development (Fig. 9C), higher expression is observed at the border region between the polyp and the stolons. With 42 hours post metamorphic development (Fig. 9D), there was strong staining in the endodermal epithelia and also in the ectodermal layer. Closer examination (Fig. 9E) showed the staining in the ectodermal layer to be in the cytoplasm of I-cells (Fig. 9F). Apically, the polyp head gave stronger staining in comparison to the other parts of the polyp. The stolon tip and the tentacles, known to comprise post-mitotic cells as in the posterior tip of the planulae, had no staining.

Within the mature colony, some staining was observed along the endodermal channels. These endothelial cells have self-renewal and proliferative capacities (Müller pers. comm.). The feeding polyps showed high intense staining along ectodermal epithelia and in the lower part of the body column. This region coincides with the position where most of the I-cells are located (Müller 1967). Their presence in this region has been confirmed by the *in situ* stainings with the probe of the *Hydractinia* I-cell marker, serum response factor (Hoffman and Kroiher 2001). In the sexual polyps, there was also some staining along the ectodermal epithelia and strong staining was observed in the vicinity of the gonophores (Figs. 10B, C), in the region where new gonophores arise and in the young developing male gonophores (Fig. 10C). In male gonophores, the period of intense staining also coincided with the period of spermatogonia proliferation. In young female gonophores, such a correlation does not exist since newly evaginating gonophores have already incorporated the final number of oocytes. However, staining observed within the young female gonophores (Fig. 17B) could be attributed to *frizzled* being a maternal message as confirmed by Northern analysis and this expression was masked by the presence of the yolk as the egg develops. There was no observable signal in the differentiated egg or sperm, while they reside in the gonophore. Yet in released eggs, *frizzled* transcripts were detected as documented in Figures 7 and 8A. Apparently the epithelial cover of the gonophores prevented staining of the oocytes.

3.2.3 Bud formation and regeneration

Studies on WNT signalling in *Hydra* indicated the major role it plays in axis formation. In *Hydractinia*, the expanding new colony is in a continual process of forming new stolons and polyp buds. One can also imagine that within its natural habitat, the colony is also subjected to predation and therefore frequently undergoes regeneration. To test the hypothesis that WNT also acts in budding and regenerative axis formation, young primary polyps were cut at the base of the polyp body and fixed at various times during the process of regeneration. As the new polyp head forms, staining was observed at the apical ectodermal tip (Fig. 11A). If the basal part of the primary polyp was still present, the polyp regenerated the missing gastric region and head. If the primary polyp was almost completely removed, the stolons, now disconnected from the source of lateral inhibition exerted by the primary polyp,

start to form polyp buds (Fig. 11B, C). Staining intensity increases with growth of the regenerating body column until the new polyp head is completely formed (Fig. 11D). In the finished head and body column, staining was fainted out.

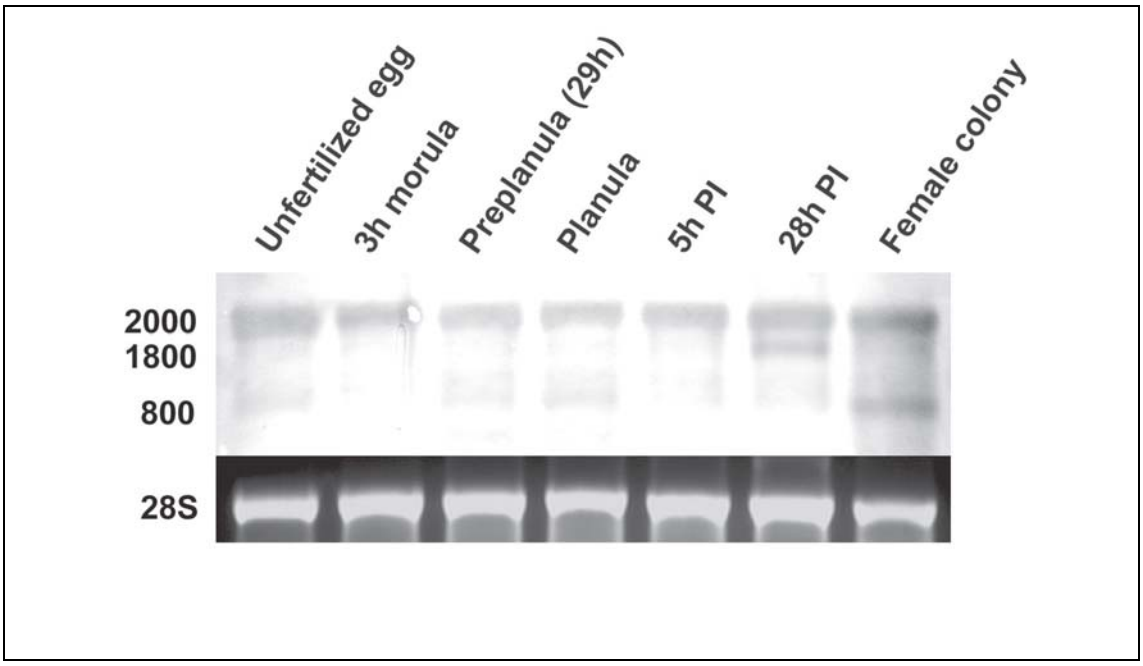

Figure 7. (Top) Northern hybridization of different stages of life cycle (Lane 3h. 29h = Approximate age in terms of hours after fertilisation; PI= post induction of metamorphosis). (Bottom) Equal loading of total RNA represented by 28S RNA.

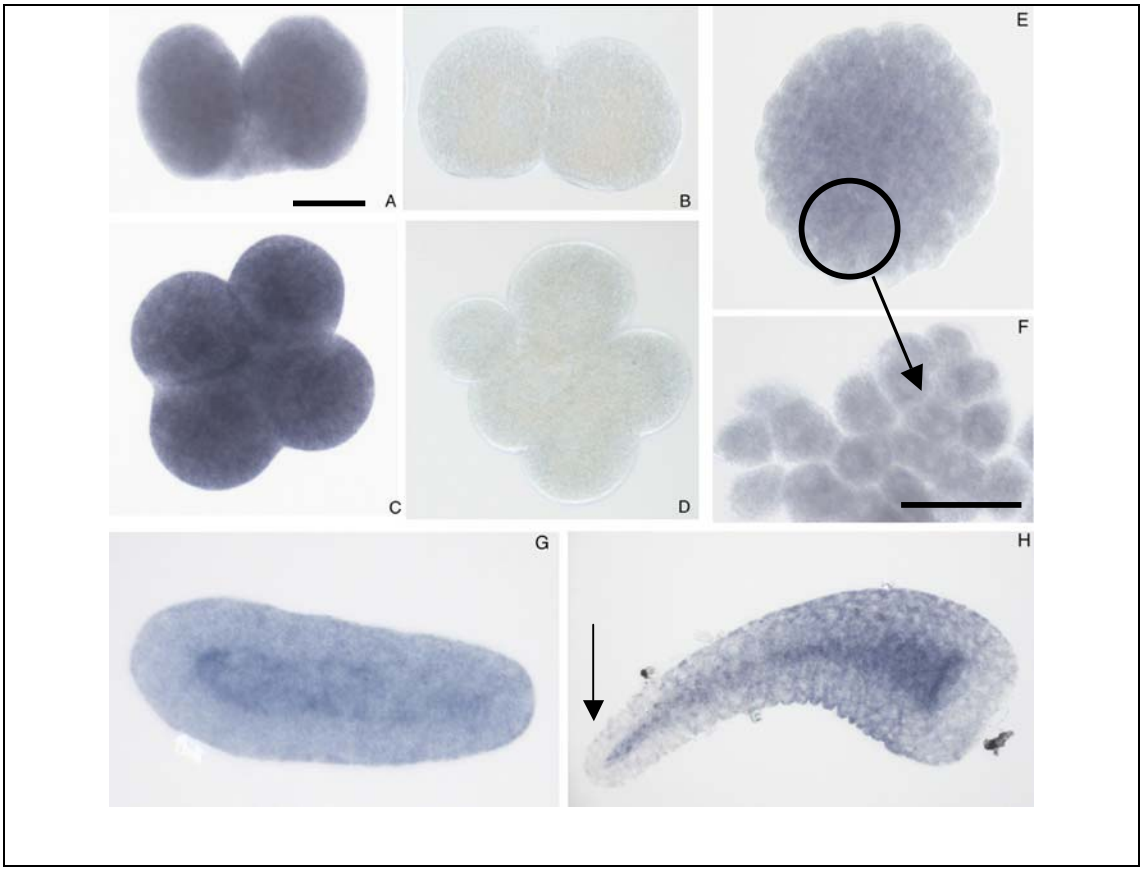

Figure 8. *He-frizzled* expression during embryogenesis. (A, C, E) Ubiquitous presence of transcript during early cleavage with expression within cytoplasm (F, arrowed). (B, D) Sense control. (G, H) Stronger expression within endodermal region of preplanula and planula larvae, corresponding with region where I-cells, nematoblasts and neuroblasts have been observed to first arise. Stinging cells at posterior end of planula larva not labelled (arrowed). Scale bar indicates approximately 100 μm.

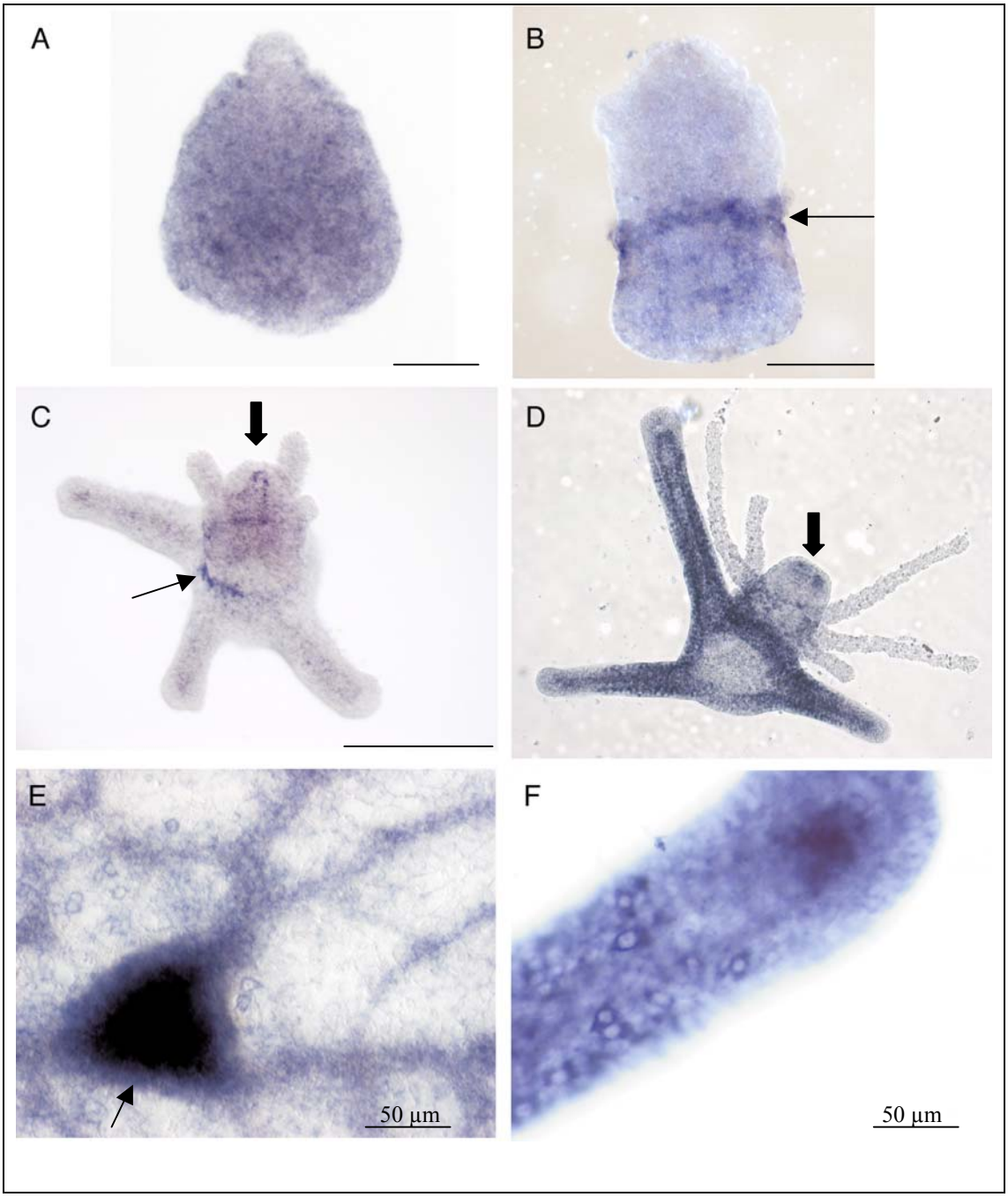

Figure 9. *He-frizzled* labelling at different time points after induction of metamorphosis. A, 5 hours after induction of metamorphosis, B. 18 hours after induction of metamorphosis, with stronger labelling at regions of higher proliferation (arrow). C. 27 hours after induction of metamorphosis. Narrow arrow points to the polyp/stolon junction where many I-cells and nematoblasts reside. The blocked arrow points to the mouth of the polyp. D. 42 hours after induction of metamorphosis, with some labelling at future mouth (blocked arrow, C-D) and along ectoderm of expanding stolon. E. Mature colony, with developing polyp bud intensely labelled (arrowed), also cells along endodermal channels and subpopulation of I-cells. F. Stolon with I-cells labelled. Scale bar indicates approximately 500 µm unless otherwise stated.

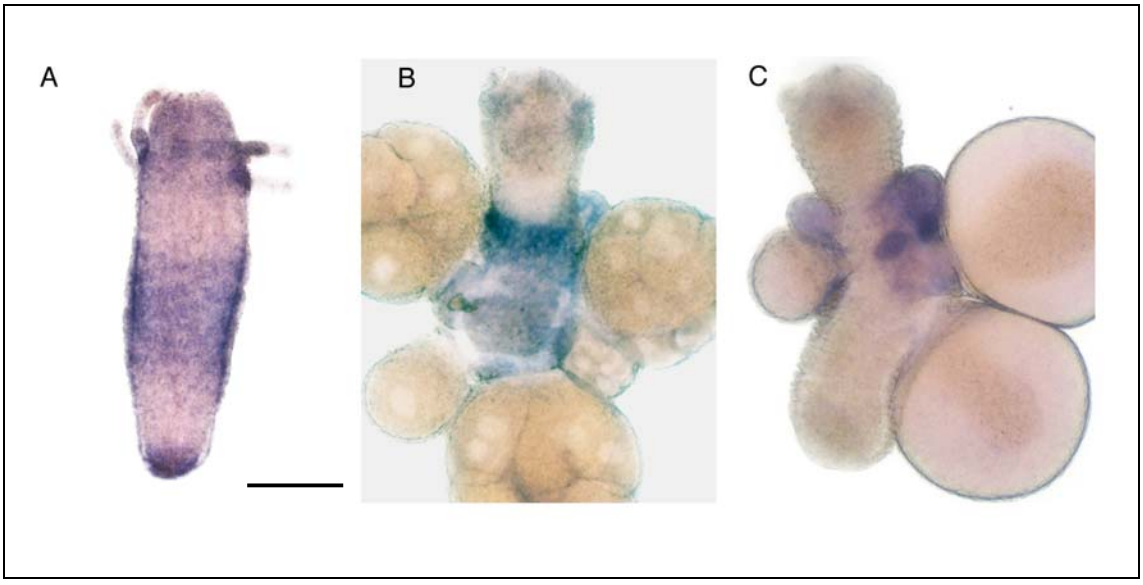

Figure 10. *In situ* labelling in polyps of mature colony. A. Feeding polyp. B. Female sexual polyp with accumulation of oogonia/oocytes stained. C. Male sexual polyps with developing spermatogonia in young gonophores stained. Scale bar indicates approximately 500 μm.

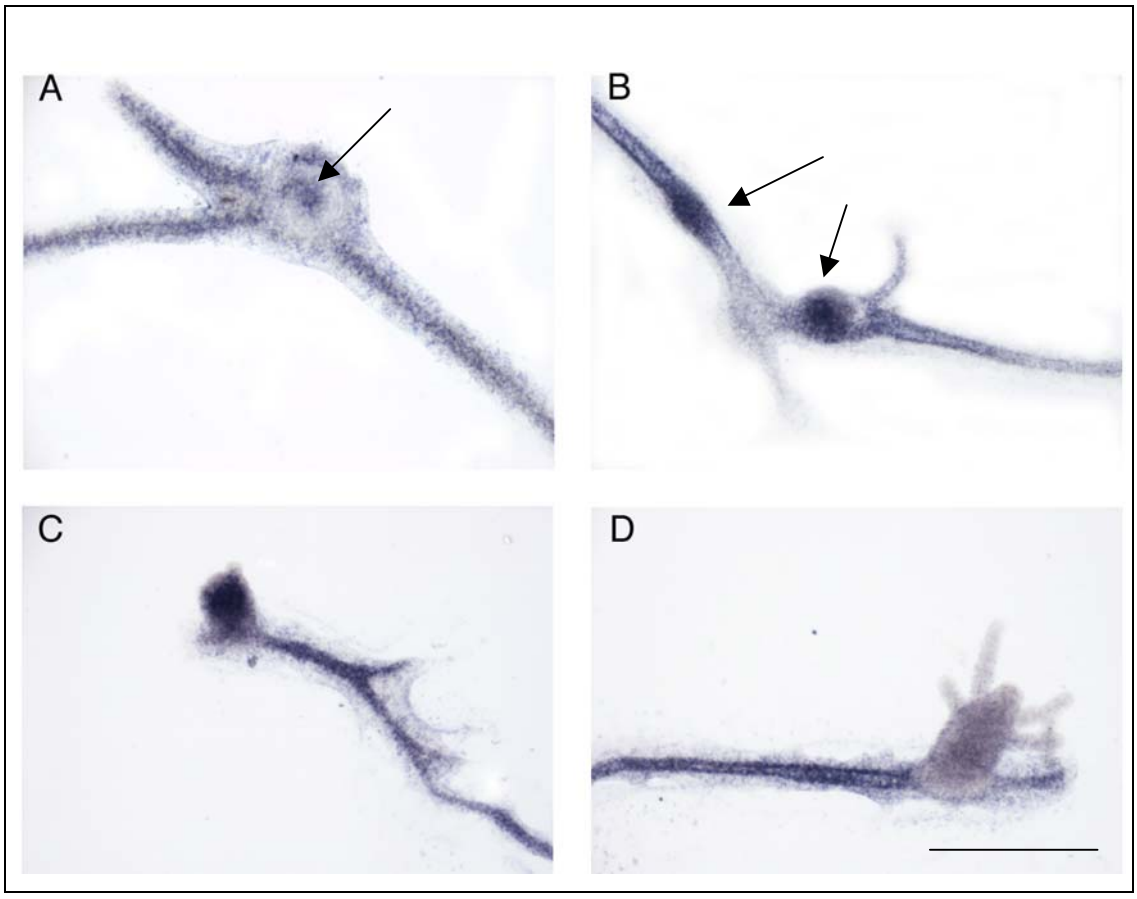

Figure 11. *In situ* labelling of young primary polyps undergoing regeneration of new polyp body. A. Faint signal at tip where new polyp will appear. B. Intense signal at regions of developing polyp. C. Developing polyp head with strong signal. D. Unobservable signal within polyp body after completion of regeneration. Scale bar indicates approximately 250 μm.

3.2.4 Frizzled expression in I-cells

Comparison of the I-cell population stained by *He-frizzled* in the stolonal mat and stolon (Figs. 9E-F) with that from conventional stainings (Fig. 12) suggests that *He-frizzled* stains only a subpopulation of I-cells. Furthermore, when comparing *He-frizzled* labelling of the I-cells in the sexual female polyp with conventional staining (Fig. 13A), *He-frizzled* labelled many cells near the vicinity of developing gonophores (Fig. 13B) but not throughout the body column (Fig. 13A). Support for *He-frizzled* labelling proliferating I-cells, oogonia and spermatogonia, comes from comparison with the probe of the germ cell marker, *vasa* (Fig. 13C), where only few cells in the (developing) gonophores were labelled.

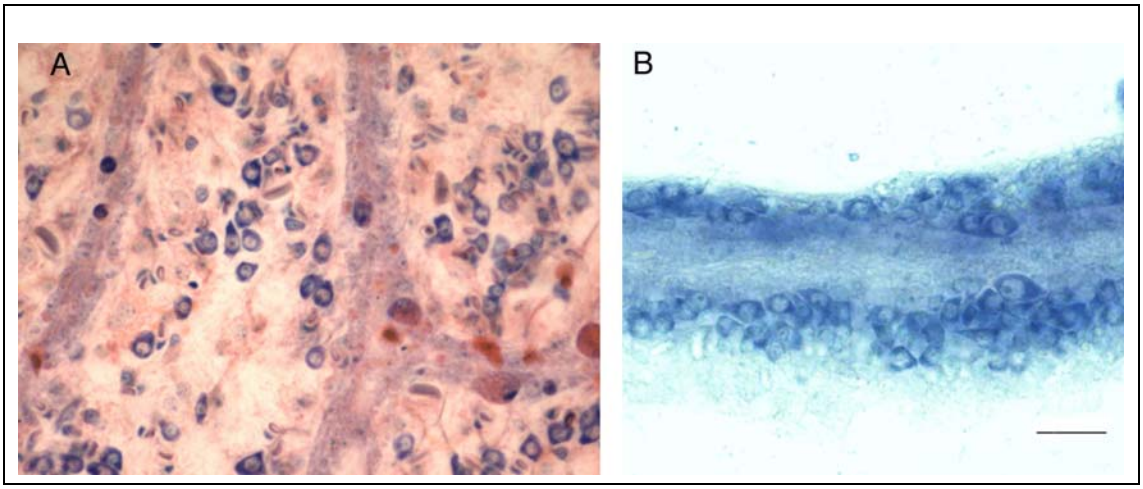

Figure 12. Stainings with Giemsa and May-Grunwald showing the normal population of I-cells stained within (A) stolonal mat region and (B) within stolon of primary polyp. Scale bar indicates approximately 50 μm.

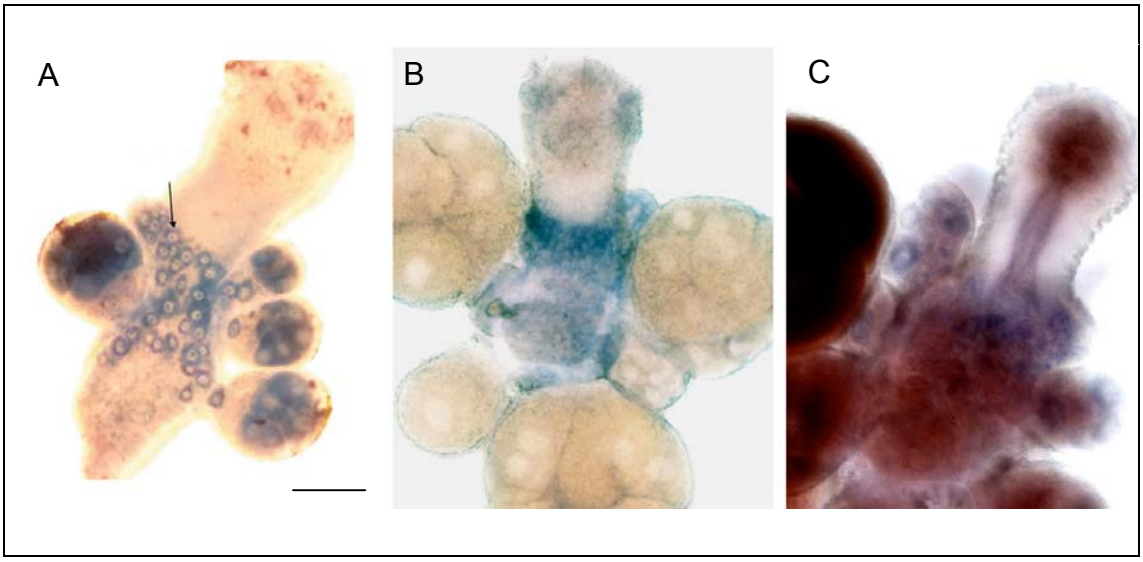

Figure 13. Comparison of I-cell stainings in female sexual gonozooids from A. Giemsa-May Grunwald, oogonia/oocytes arrowed. B. *In situ* probe *frizzled*. C. *In situ* probe of germ cell marker *vasa*. Scale bar approximately 500 μm.

3.3 Effects of using GSK-3 inhibitor

3.3.1 Morphogenesis affected with alsterpaullone and azakenpaullone

Two days after overnight treatment with a low dose (0.10 μM) of **alster**paullone, a large increase in the number of I-cells was observed in the mature colony (Figs. 14A, B). Approximately within the same time period, the stolons and areas of the stolonal mat at the periphery of the colony started to degenerate (Fig. 14C, arrowed). With time, large buds started to form, and from these, new polyps arose (Figs. 14D, E). The size of the giant buds surpassed the size of normal buds several hundred fold. They gave rise to several, frequently multiheaded polyps (Fig. 14E).

Planulae treated overnight with various **azaken**paullone concentrations and subsequently induced to metamorphoses showed that over a 3-day period, the treatment resulted in lower number of stolon outgrowths (Fig. 15A). At a higher concentration (0.10 μM) of azakenpaullone, initial growth of stolons was even suppressed (Fig. 15A). Tentacle growth also showed similar suppression at higher concentration of 0.10 μM compared to controls or at concentrations of 0.05 μM, however with 0.05 μM azakenpaullone treatment, higher tentacle numbers (Fig. 15B) and also a higher percentage of ectopic tentacles (Fig. 15C) resulted after three days. Higher doses of azakenpaullone above 0.20 μM resulted in no growth of stolons and tentacles, and sometimes caused damage to the planulae that led to death.

3.3.2 Interstitial stem cell population affected with alsterpaullone

Comparison of the effects of alsterpaullone on the differentiated products of the I-cell population indicated that there was a significant difference in the number of nerve cells three days after treatment with 0.1 µM alsterpaullone (Figs. 16A, B, 17C). At six days after treatment, there was also a significant increase in the number of nematocytes (Figs. 16C, D, 17D). All tests were significant at $P<0.001$ and carried out with the Mann-Whitney U Test.

Since such large number of nematocytes cannot come from the terminal differentiation of committed I-cells alone, there must be a period of proliferation before such large numbers of differentiated products can be observed. To test this assumption, BrdU labelling was carried out and a significant number of cells was observed to undergo proliferation (Fig. 17A), following six hours after treatment. Furthermore, proliferating cells were even observed at the stolon tips (Fig. 16F), an event that has never been observed in non-treated animals (Fig. 16E).

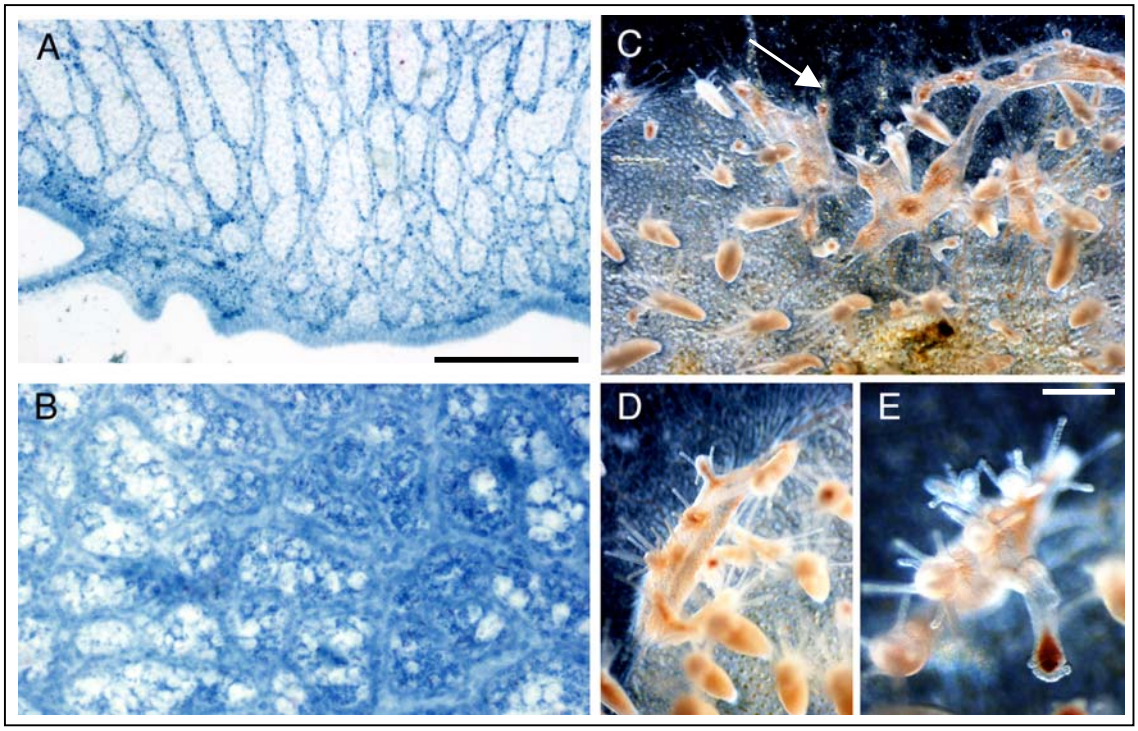

Figure 14. Effects of alsterpaullone treatment (0.1 µM, 20 h) on mature colony. I-cell population in (A) control (B) two days after treatment, show dramatic increase. (C) Alsterpaullone (overnight treatment with 0.1 µM) caused cells at edge of colony to degenerate in the first few days after the start of treatment. Subsequently growth rate was also slowed. (D, arrowed) Formation of giant buds, which normally gave rise to more than one head (D, E). Scale bar indicates approximately 500 µm

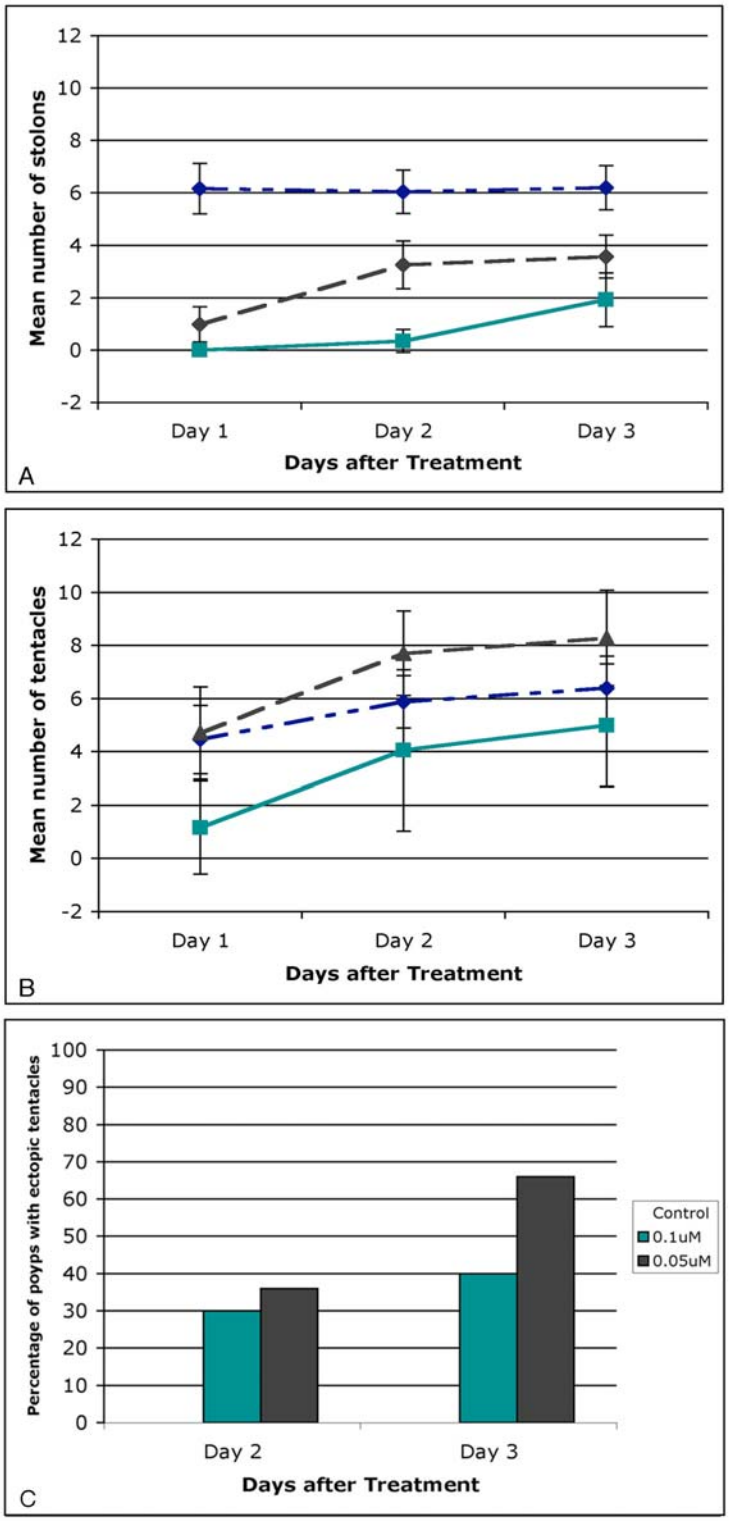

Figure 15. Effects of azakenpaullone treatment on stolons and tentacles of primary polyps. Treatment started in early metamorphosis (4h after induction) and lasted up to 3 days. N = 50 per treatment. A. At 0.10 μM, growth of stolons was suppressed, but at 0.05 μM, steady increase in mean stolon numbers. B. 0.05 μM caused more tentacles to develop per polyp in comparison to 0.10 μM. C. Higher occurrence of ectopic tentacle formation with 0.05 μM.

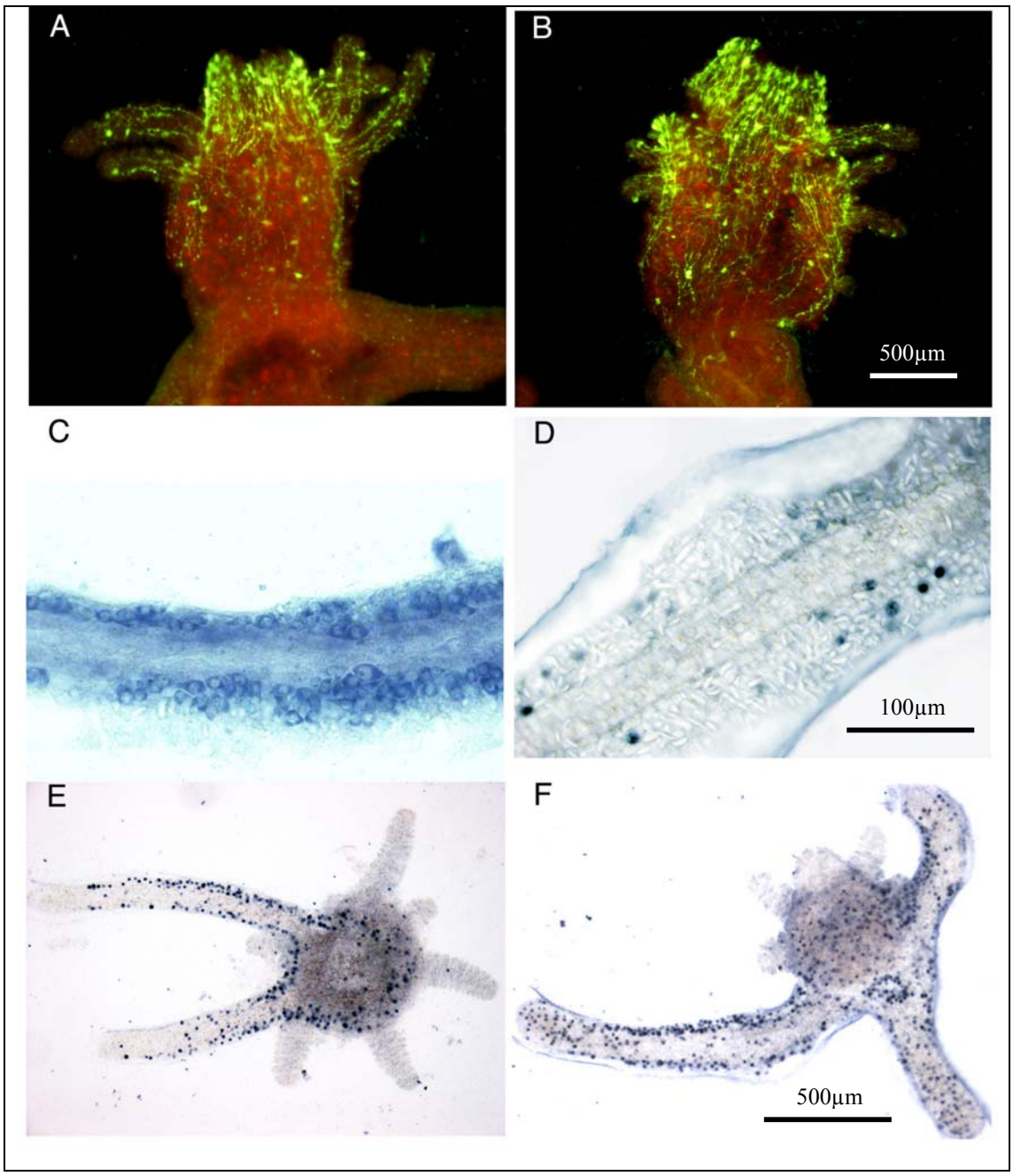

Figure 16. Effects of alsterpaullone treatment showed significant increases in derivatives of the I-cell populations. A. Nerve cells, control. B. Treated (0.125 µM, 38h; counted 3 days after treatment). C. I-cells in stolon of control were many compared to D. treated (0.25 µM, 23h, fixed four days later) where the ectodermal layer of the stolon was filled with only nematocytes, such that epithelial cells were displaced. E. Proliferating cells fewer in control than in F. treated (0.20 µM, 14h, fixed six hours later). In treated primary polyps, proliferation was also observed at stolon tips.

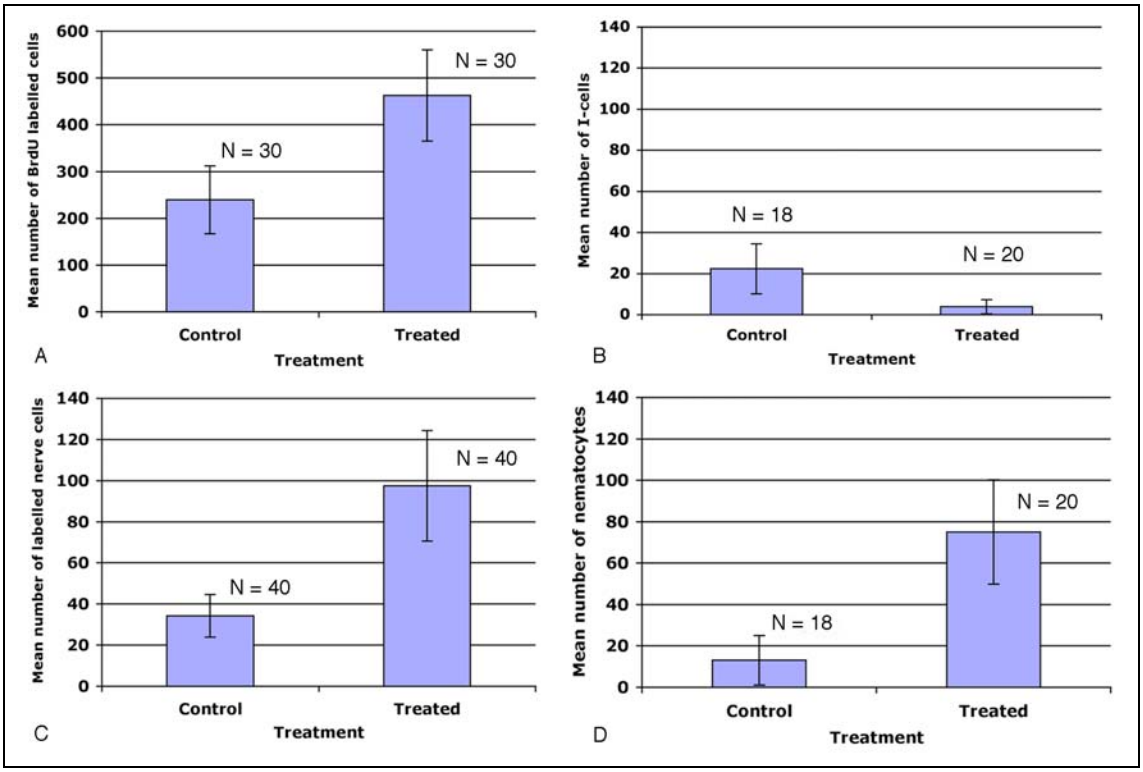

Figure 17. Mean number of cell types after alsterpaullone treatment (detailed specification of treatments in the legends of Fig. 14 to 16). All show significant differences at $p < 0.001$ with the Mann-Whitney U-Test. (A) High number of cells undergoing proliferation in the stolons of primary polyps following six hours after treatment, followed by concomitant increase in the stem cell derivatives of (C) the nerve cells after three days and (D) the nematocytes per unit area (2.31 mm^2) after six days. (B) I-cell numbers decreased with time, here shown at six days. BrdU lablled cells were counted in the stolons of primary polyps, the number of nematocytes and of I-cells refer to unit area (2.31 mm^2) of the stolon plate of colonies. The number of nerve cells refers to RF-amide positive cells in the body column and head of primary polyp.

Table 1. Table giving mean number of nematocytes and I-cells per unit area (2.31 mm^2) of the stolon plate of colonies in control and treated colonies. Subcloned colonies of the clone male 1 were treated overnight with 0.1 µM for 18h. This treatment was repeated for 4 successive days. The data were counted at day 6, i.e. 2 days after the last treatment. Means are given with standard deviation. In a second set of experiments, the animals were treated once with 0.25 µM, 15h. Since the values counted for these experiments were similar, they were summarised with those from the first experiment. Ratio indicates number of nematocytes to I-cell.

	I-cells total counted	I-Cells: means per unit area	Nematocytes total counted	Nematocytes means per unit area	Ratio
Control	402	22.3 +/- 12.2	235	13.1 +/- 12	0.59
Alsterpaullone-treated	78	3.9 +/- 3.5	1500	75.0 +/- 25.2	19.23

Comparison of the ratio of I-cells to the nematocytes between control and treated colonies at 6 days later, indicated that there was more than a 19-fold increase in the number of nematocytes in the treated (Table 1). Furthermore, the after Mann-Whitney U Test indicated that the increase in the number of nematocytes in the treated samples compared to the non-treated controls was highly significant with $p<0.01$. In addition, a chi-squared comparison of the ratios calculated an even higher significance with $p<0.001$.

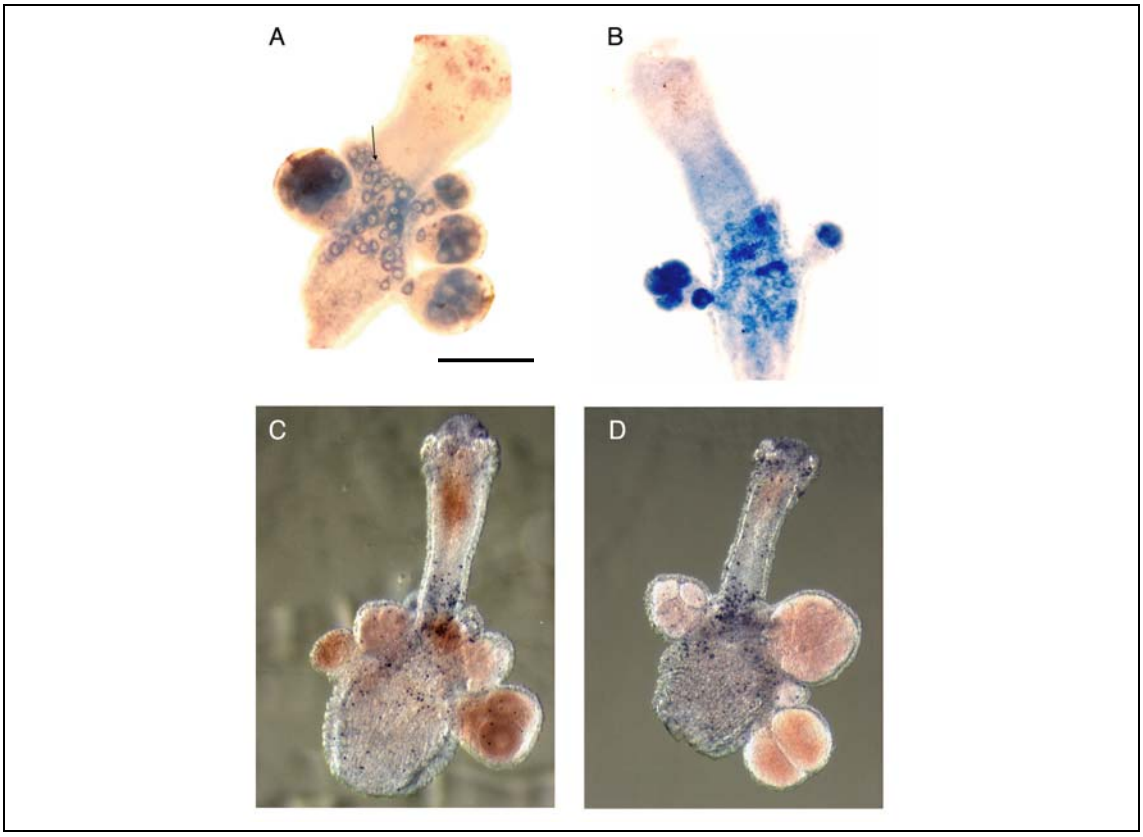

Figure 18. Opposite effects on female germ cells after alsterpaullone treatment as observed in nerves and nematocytes. A. Large number of normal-sized oocytes/oogonia in control (arrowed). B. Few oogonia/oocytes observed. No observable difference observed in proliferating cells between C. control and D. treated (0.10 μM, 67-day period; 3 days treatment followed by 2 days no treatment). Scale bar indicates approximate 500 μm.

Long-term treatment with alsterpaullone (0.10 μM) on mature colonies resulted in slower growth and later development of sexual gonozooids (data not shown). Comparison of the presence of oogonia/oocytes in control and treated (Figs. 18A-B), showed fewer number of normal-sized oogonia/oocytes in the treated. BrdU labelling of the gonozooids did not indicate any observable differences (Figs. 18C-D).

Discussion

Of the WNT family of proteins within the cnidarians, there is at least one member in *Hydra* (Hobmayer et al., 2000) and ten in the anthozoan *Nematostella vectensis* (Kusserow et al., 2005). Support on the conservation of the evolutionary function of the proteins within the WNT canonical cascade comes from injection studies of transcripts of *Hydra ß-catenin* in *Xenopus* embryos (Hobmayer et al., 2000) and *Hydra GSK-3ß* in zebrafish embryos (Rentzch et al., 2005). These transcripts both caused the classical morphogenetic effects expected as when injected with the animal's own mRNA, i.e., secondary axis formation in *Xenopus* and ventralisation in zebrafish. Currently, only *Hydra* has the most components (*Wnt*, *Frizzled*, *GSK-3ß*, *ß-catenin*, *Tcf*) within the canonical WNT pathway identified (Hobmayer et al., 2000, Minobe et al, 2000).

In general, WNT signalling has been implicated in many processes in embryogenesis. (For general overview of functions, refer to website: http://www.stanford.edu/~rnusse/WNTwindow.html). The most well-known of its functions is the determination of one of the future axes of bilaterian animals. This function is attributed to the canonical WNT pathway and is associated with WNTs from Class 1, these comprising WNT1, -3, -8a, -8b.

A second known function is the polarizing effect of WNT signals in epithelia, in the planar cell polarity (PCP) pathway. In epithelia, WNT signalling directs the orientation of multiple elementary structures and outgrowths such as cilia, bristles and hair follicles. Present literature attributes this function to a non-canonical β-catenin-independent WNT pathway that also requires the protein Dishevelled (Strutt 2003).

A third known function is the role of WNT5A and WNT11 in cell movement, in particular in gastrulation movements of *Xenopus* embryos, including convergent extension movements and separation of germ layers. This function is also attributed to a non-canonical, β-catenin-independent pathway, known as the WNT/Ca^{2+} pathway (Kuhl et al., 2000).

A large group of WNTS appear to be involved in stem cell fate decision in mammals and other vertebrates. This group comprises the same WNT isoforms involved in axis specification, and they signal via GSK3β, along the β-catenin route. This canonical aspect will be further discussed below.

I address the question whether the effects described here can be attributed to the canonical pathway and specifically its role in stem cell fate, as *Frizzled* expression in *Hydra* has been hypothetically been associated with planar polarity (Minobe et al., 2000) however without any experimental evidence.

Furthermore, the chemicals used for the functional studies constitute a class of small molecules termed paullones, shown to be potent inhibitors of the glycogen synthase kinase-3 (GSK3) but also of several cyclin-dependent kinases (CDK) (Table 2). GSK3β is not only a known component of the canonical WNT pathway but also of other pathways e.g. Hedgehog pathway, and inhibition of GSK3β along any of these pathways could lead to the induction of cell proliferation. Given the known targets of paullones and the known substrates of GSK3β, the extent to which the results obtained from the current functional studies are a direct effect on the WNT canonical pathway has also to be addressed.

4.1 *He-frizzled1* shares domains present in other Frizzleds and has motif required for canonical WNT signalling

The *He-frizzled1* sequence obtained gave a predicted protein structure typical to all other known Frizzleds with a cysteine-rich domain (CRD) and a putative seven transmembrane region. The presence of the motif KTXXXW after the last transmembrane region supports the functionality of this gene in the WNT/ß-catenin signalling (Umbhauer et al., 2000). Its close relationship to the Frizzled-7, points to the possibility of all three pathways existing within *Hydractinia*.

The high homology to the *Hydra frizzled* supports the conservation of *He-frizzled1* within Hydrozoa. In the phylogram obtained, the Hydrozoa cluster forms a group with the vertebrate Frizzled-2, -7 and –10. This corresponds to the results obtained from BLAST searches of the cnidarian *Acropora* genes, where higher overall similarity has been observed to the corresponding human sequences. The current result supports the hypothesis of less gene loss or sequence changes between cnidarians and vertebrates from the ancestral urbilaterian ancestor compared to *Drosophila* or *C. elegans* (Kortschak et al., 2003).

Since the *He-frizzled1* sequence corresponds to the largest band observed in the Northern analysis, all subsequent usage of this name is in reference to the largest band, if not stated otherwise.

4.2 *He-frizzled1* in embryonic and post metamorphic stages

Current data suggest the close relationship of *He-frizzled1* to the vertebrate *frizzled* 2 and 7. Known functions elicited by these two proteins compared to the possible roles of the different *frizzled* genes (In the current context, I will assume there are three genes in *Hydractinia*) within *Hydractinia* will be considered.

The Northern and *in situ* analyses conducted were carried out with a probe within the transmembrane region. This suggests that the *in situ* results could show the expression pattern of any of the three genes at any point in the life cycle of *Hydractinia*. The Northern analysis points to the presence of which gene but not to the pathway that is being activated. Activation of any of the WNT pathways is exclusive to the specific WNT-Frizzled combination, but the Frizzled receptor is not exclusive to any pathway. Frizzled-7 has been shown to function in both canonical and non-canonical pathways (Swain et al., 2001). Hence one receptor has the potential to transduce any of the pathways, and effect different functions.

In *Hydra*, the Northern and Southern analyses indicated the presence of only one *frizzled* gene (Minobe et al., 2000), however there are three putative forms existing within *Hydractinia*. How these three function within *Hydractinia* and which pathway they elicit can be presumed only by extrapolation to the known functions within other organisms or in cultures of mammalian cells. Comparisons with the life cycle of *Hydra* could also explain the presence of the different *frizzled* products observed at the various stages of *Hydractinia*.

Common to both *Hydra* and *Hydractinia* is the continual renewal of products from the mulipotent I-cell lineage and regeneration/growth of new polyp buds. Since the major bands from *Hydra* and *Hydractinia* are similar in size (approximately 2000 bp), these two *frizzled* products possibly serve similar functions within *Hydra* and *Hydractinia* respectively and this main band possibly correspond to function within cells anticipating proliferation, and perhaps also play a role in the maintenance of the I-cell population. The lack of expression observed from the fully developed oocytes in the gonophore could be due to the inability of the RNA probe to bypass the envelope surrounding the mature gonophore or its presence was masked by the large amount of yolk as the Northern analysis already showed the high levels of this transcript as a maternal message. Moreover, transcripts were present in early embryos before zygotic expression is known to commence. The band observed at 1800 bp, at 28 hours post

induction of metamorphosis, could possibly relate to the gene responsible for the formation of new structures, such as oral structures. Comparison with stages from the *in situ* analyses (Figs. 9C, D) showed that at these stages, the young polyp head is already formed but there is a higher expression at the oral tip nonetheless. This could possibly relate to the formation of the new mouth opening and the determination of the sensory cells that surround the opening.

Given that the RNA probe was from the transmembrane region, the small band at approximately 800 bp could correspond to a polypeptide that spans the entire transmembrane region only. It has been mostly accepted that the cysteine-rich domain (CRD) is required for WNT binding and subsequent transduction of the signal. However in the human (interleukin) IL-8 receptor, the N-terminal domain which bound ligands with high affinity was found to be dispensable and that the extracellular loops linking the transmembrane regions that appeared to be critical for receptor activation (Ahuja et al., 1996). Thus this 800 bp band could represent a protein not only capable of binding WNTs at the extracellular loops of the transmembrane region, but also of transducing the signal.

Northern analysis showed that this band first appeared at the 29-hour embryo (preplanula), coinciding with the time period when gastrulation is known to take place (Eiben 1982). This may function to act either as a prelude to the formation of new cell types, e.g., I-cells and their derivatives, thereby explaining the stronger *in situ* staining within the central endodermal mass of the preplanula and planula, or for purposes of patterning, and tissue separation (Winklbauer et al., 2001), functions shown to be essential during *Xenopus* gastrulation.

In situ staining of the I-cells in the developing primary polyp and within the stolonal compartment of the mature colony suggested that *frizzled* was expressed in a subpopulation of I-cells only, especially when comparing the density of I-cells stained by conventional staining. Since I-cells do not undergo continuous proliferation and together with the observations of the intense ubiquitous staining from the pre-gastrula stages of *Hydractinia*, these suggest that *frizzled* probably prepares cells to receive proliferation-stimulating signals. Furthermore, comparison of the *in situ* staining within the sexual polyps with that from *vasa* (Fig. 13) showed that *frizzled* did not stain all germ cells, only a subpopulation. Taken together, it appears that *frizzled* prepares cells for proliferation, and this is not confined to any specific cell types. On

the other hand, not all cells expressed *frizzled* to the same extent. Generally ectodermal epithelial cells were not, or only faintly, stained by *in situ* hybridisation. In addition, not all cells responded to GSK3ß inhibition in the same way. While the population of nerve and stinging cells were caused to increase, there was no similar increase in the polyp's body column, stolons or tentacles observed. The effects of GSK3ß inhibition will be discussed in more detail below.

Results from the regenerating polyp head and new bud formation support the classical function of WNT signalling in axis formation. Higher intensity at the developing polyp head could be due to the combination of signals for axis formation, cell proliferation and formation of new structures. This probably accounts for the ubiquitous stainings observed also in the developing primary polyp after metamorphosis.

4.3 Effects of using alsterpaullone and azakenpaullone

Table 2 lists the IC_{50} values of alsterpaullone and azakenpaullone on the inhibition of CDK/cyclin, LCK and GSK3 at values of less than 1 μM. Work by Leost and co-workers (2000) showed that there was an almost 10-fold effect of paullones inhibiting GSK3 than CDK1 or CDK5. In contrast, Bain et al., (2003) showed that GSK3ß and CDK2/CyclinA had similar concentrations for alsterpaullone, while azakenpaullone showed a 3-fold higher inhibitory effect for GSK3ß. A study conducted to identify intracellular targets of paullones using affinity chromatography confirmed the high affinity of paullones to both isoforms of GSK3 (GSK3α and GSK3ß), but also resulted in the identification of another target, mitochondrial malate dehydrogenase (MDH) (Knockkaert et al., 2002), however the IC_{50} values for MDH were above 2.0 μM, at concentrations that are over 2.5-500x higher than those required for the inhibition of GSK3 or CDK.

The most obvious effects of pulse treatment with alsterpaullone (0.10-0.25 μM) and azakenpaullone (0.05-0.10 μM) in the current study were the formation of many, and giant buds, and polyps with ectopic tentacles or multiple heads in the mature colony, the proliferation of I-cells and with time, a significant increase in the number of terminally differentiated I-cell derivatives (Figs. 14, 16) like the nerve and stinging cells. With long-term daily alsterpaullone (0.10 μM) treatment in the mature colony, slower growth was observed compared to controls (not shown), including

slower development of gonozooids and gonophores, and fewer germ cells were observed (Fig. 18). Given the use of azakenpaullone and alsterpaullone in this study, the question is then how these chemicals could act on CDK and GSK3 such that we observe the current phenotypes. The action of each of the inhibitors on each of these enzymes will be examined.

Table 2. Studies showing concentration of alsterpaullone and azakenpaullone required for 50% inhibition (IC_{50}) of the various protein kinases. Only enzymes with concentrations of less than 1.0 µM shown. CDK = cyclin-dependent kinase, GSK = glycogen synthase kinase, LCK = lymphocyte kinase, p = phosphatase, ND = not determined.

Enzyme	**Alsterpaullone IC_{50} (µM)**	**Azakenapullone IC_{50} (µM)**	**Reference**
CDK1/CyclinB	0.035	0.400	Schultz et al., 1999
CDK2/CyclinA CDK5/p35	ND	0.680 0.850	Zaharevitz 1 et al., 1999
GSK3 CDK1 CDK5	0.004 0.035 0.040	0.023 0.400 0.850	Leost et al., 2000
GSK3ß CDK2/CyclinA LCK	0.110 0.080 0.470	0.230 0.670 0.470	Bain et al., 2003
GSK3ß CDK1/CyclinB CDK5/p25	ND ND ND	0.018 2.000 4.200	Kunick et al., 2004

4.3.1 Action of inhibitors on CDK

In general, paullones are known to inhibit CDKs by arresting the different enzymes at various stages of the cell cycle and their anti-tumour activity *in vitro* has also been tested and confirmed (Schultz et al., 1999, Zaharevitz et al., 1999). Both alsterpaullone and azakenpaullone have been found to be potent and ATP-competitive

inhibitors of GSKs-3 and CDKs, with azakenpaullone having a 100-200 fold greater selectivity for GSK-3ß over Cdk1/B and Cdk5/p35 (Kunick et al., 2004).

Cell cycle effects of azakenpaullone have been carried out using MCF10A breast epithelial cell lines, and at a concentration (30μM) where 50% of growth was inhibited, these cells were arrested at the G_1/S boundary (Zaharevitz et al., 1999). Antipoliferative activity of paullones conducted on the colon cell lines (HCT-116 line shown) indicated a noteworthy antitumour activity (Schultz et al., 1999). This study also reported that good inhibition of kinase activity did not necessarily translate to good antitumour activity.

In the current study, **azaken**paullone was used only to study its effects on the metamorphosing primary polyp. This substance was a kind gift from Dr. Conrad Kunick (Technical University of Braunschweig, Germany), and the amount was sufficient only for this study. It appeared that azakenpaullone did affect cell cycle progress within the stolons and at 0.10 μM, also that of tentacles. However at 0.05 μM, there was an obvious increase in percentage of ectopic tentacles (Fig. 15C). Thus on the one hand there was the growth suppression of stolonal cell types, but an opposite effect for tentacles. Although azakenpaullone has a 100-200 fold greater selectivity for GSK-3ß over Cdk1/B and Cdk5/p35 (Kunick et al., 2004), most literature discussed the inhibition of GSK-3ß as leading to cell proliferation effects (see Table 3), therefore it appears that azakenpaullone (especially at higher concentration of 0.10 μM) affected the CDKs by suppressing stolon and tentacle growth, but also affected GSK-3ß at lower concentrations of 0.05 μM, possibly via the canonical WNT pathway, thereby leading to increased tentacle numbers and ectopic growth (Fig. 15B).

Experiments using **alster**paullone on the adult colony gave ectopic heads (Figs. 14C-E) and two days after treatment, a large number of I-cells and derivatives were also observed in the stolonal mat (Figs. 14A-B).

Within the primary polyp, as early as six hours after overnight alsterpaullone treatment, there was already a significant increase in the number of proliferating cells (Fig. 17A); three and six days after treatment, there were significant increases in the number of nerve cells and nematocytes respectively (Figs. 16B, D, 17C-D). In these experiments, if alsterpaullone did inhibit the activity of CDKs in *Hydractinia*, then one would observe instead no increase in any cell types. Moreover, the more specific

CDK inhibitor aminopurvanolol did not cause an increase in these cell types (Müller, pers. comm.). Hence, it appears that there was minimal effect of alsterpaullone on the CDKs in *Hydractinia* given the low concentration used and alsterpaullone possibly activated the canonical WNT pathway and caused increased cell numbers. However, at higher concentrations, which caused a stop to growth and development, inhibition of CDKs may well have contributed to the deleterious effect.

4.3.2 Action of inhibitors on GSK3

Growing interest in developing inhibitors for GSK3 arise from the realisation that GSK3 is involved in many pathways leading to pathologies like diabetes, Alzheimer's disease, stroke and bipolar disorder. Many compounds have now been developed to target GSK3 for therapeutic purposes, however due to the multifunctionality of GSK3, selectivity for the treatment is questionable.

The chemical variety of the inhibitors is wide, but all share similar features. They are flat, hydrophobic structures with low molecular weight and interact with GSK3 in similar ways (review Meijer et al., 2004). Kinetic studies of alsterpaullone have shown that it competes actively with ATP for binding to its substrates (Leost et al., 2000). No similar study has been conducted with azakenpaullone but kinetic studies with kenpaullone (both chemicals having the same parent structure) showed that it acted also by competitive inhibition of ATP binding to its substrates (Zaharevitz et al., 1999) and in the case of GSK3, this inhibition prevented GSK3 from phosphorylating its substrates.

Current evidence implicating the role of GSK3ß in axis formation is its role within the canonical WNT pathway. This same pathway has also been implicated in functions related to self-renewal in haemopoietic stem cells (Reya et al., 2003). In order to activate the canonical WNT pathway to elicit any of these functions, inhibition of GSK3ß (Figure 1) is required. By using specific GSK3ß inhibitors like azaken- and alsterpaullone, the activation of the canonical WNT pathway is being simulated.

The pulse treatment of alsterpaullone did lead to bud and ectopic axis/head formation, this strongly suggests that the canonical WNT pathway was activated within *Hydractinia* in order to obtain such phenotypes. The evidence for the existence of this pathway comes from injection studies in the cnidarian *Hydra*. Injection into

ventral blastomeres of *Xenopus* embryos at the eight-cell stage with the *Hydra* ß-catenin mRNA gave embryos that subsequently developed secondary axes (Hobmayer et al., 2000), while microinjection of *Hydra* GSK3ß transcripts inhibited ß-catenin in zebrafish (Rentzsch et al., 2005). Furthermore, studies by Müller and co-workers (2004b) have already showed that alsterpaullone can effect multiple head formation in *Hydractinia*.

Table 3. List of protein phosphorylated by GSK3ß and the pathway initiating the action. All proteins here have the potential to cause proliferation of cells when GSK3ß fails to phosphorylate them. Phosphorylation (*) leads to ubiquitylation and subsequent degradation by proteasome.

Protein phosphorylated by GSK3ß	**Pathway involved in inhibition of GSK3ß**	**Function when dephosphorylated**	**Reference**
APC	Wnt	Activation of transcription e.g. cyclin D and c-Myc	Ikeda et al., 2000 He et al, 1999 Shtutmann et al., 1999
Axin	Wnt	Activation of transcription e.g. cyclin D and c-Myc	Ikeda et al., 2000 He et al, 1999 Shtutmann et al., 1999
*ß-catenin	Wnt	Activator of transcription e.g. cyclin D and c-Myc	Ikeda et al., 2000 He et al, 1999 Shtutmann et al., 1999
*c-Myc	PI_3K-Akt	Transcription factor activated	Dominguez-Caceres et al., 2004
Cubitus interruptus	Hedgehog	Activation of transcription e.g. cyclin D and E	Jia et al., 2002, Duman-Scheel et al., 2002
*Cyclin D	PI_3K-Akt	Transcription factor activated	Diehl et al., 1998
NFATc		Transcription factor translocated out	Crabtree and Olsen 2002

Table 3 lists the proteins that are substrates of GSK3ß and inhibition of GSK3ß action on any of these substrates has already been shown to result in cell proliferation. In fact, mutations found in the listed protein substrates have already been implicated in various forms of cancer. Hence although it appears that the

canonical WNT pathway is activated within the current study, the inhibition of GSK3ß on any of the other substrates may also lead to the observed proliferation of cells. Therefore, one cannot exclude the possibility that besides the canonical WNT pathway, any of the above pathways could have also resulted in the proliferation of cells. However, it has been shown that treating gastrulating embryos with alsterpaullone, azakenpaullone or lithium, resulted in bipolar, double-tailed or multipolar/multi-tailed planulae larvae (Müller and Plickert pers. comm.), and anti-β-catenin immunocytochemistry of these treated primary polyps showed the presence of many cells with I-cell like morphology with high expression of β-catenin compared to controls (Müller pers. comm.), all further suggesting that the WNT canonical pathway was activated upon alsterpaullone blocking of GSK-3β.

4.3.3 Other effects of using alsterpaullone

Within the hydrozoan *Podocoryne*, homologues of the CCAAT/enhancer binding protein (C/EBP) has been located, and *in situ* labelling has localised their expression to the mature gonads (Seipel et al., 2004b). Homologues of this protein have already been found within the *Hydractinia* ESTs. Furthermore, a study conducted by Ross and co-workers (2000) has shown that WNT signalling results in the inhibition of adipogenesis, with a loss of C/EBPα expression. Hence the slow development of the gonophores in the long-term treatment could have been the result of the loss of expression C/EBP, thereby affecting gametogenesis.

In Jurkat cell lines, it has been observed that use of alsterpaullone can induce apoptosis (Lahusen et al., 2003). Similarly, long-term usage of alsterpaullone could have induced the high rates of apoptosis observed with initial treatments (Fig. 14C), and subsequently also affected growth rates.

4.4 Stem cell fate

Current literature on the role WNT canonical pathway plays on stem cell fate appears controversial. Some attribute its role in stem cell renewal and expansion (Ikeya et al., 1997, Chenn and Walsh 2002, 2003), while others have shown its role in the specification of cell fate and in guiding cells out of the cell cycle into terminal differentiation e.g, neural crest stem cells (Lee et al., 2004). However similar studies in invertebrate stem cells is so far unknown.

The current study showed that with short-term blocking of GSK-3β, there was an initial burst of S-phase activity followed by a significant increase in the number of differentiated nerve cells and nematocytes, however subsequently with a low number of typical I-cells (Fig. 17). This suggests that in *Hydractinia*, activating the WNT pathway does not cause an expansion of the totipotent I-cell population, but those of the progenitors, leading first through a phase of proliferation, and subsequently to terminal differentiation (Fig. 19). This follows the paradigm of erythropoietin (epo) in the haematopoietic system of mammals where, upon stimulation by epo, such descendants first undergo a certain number of divisions before proceeding to terminal differentiation. Similarly, the observed increase in the nerve cell population also has parallels in the mammalian system (Ikeya et al., 1997, Chenn and Walsh 2002, 2003, Castelo-Branco et al., 2004).

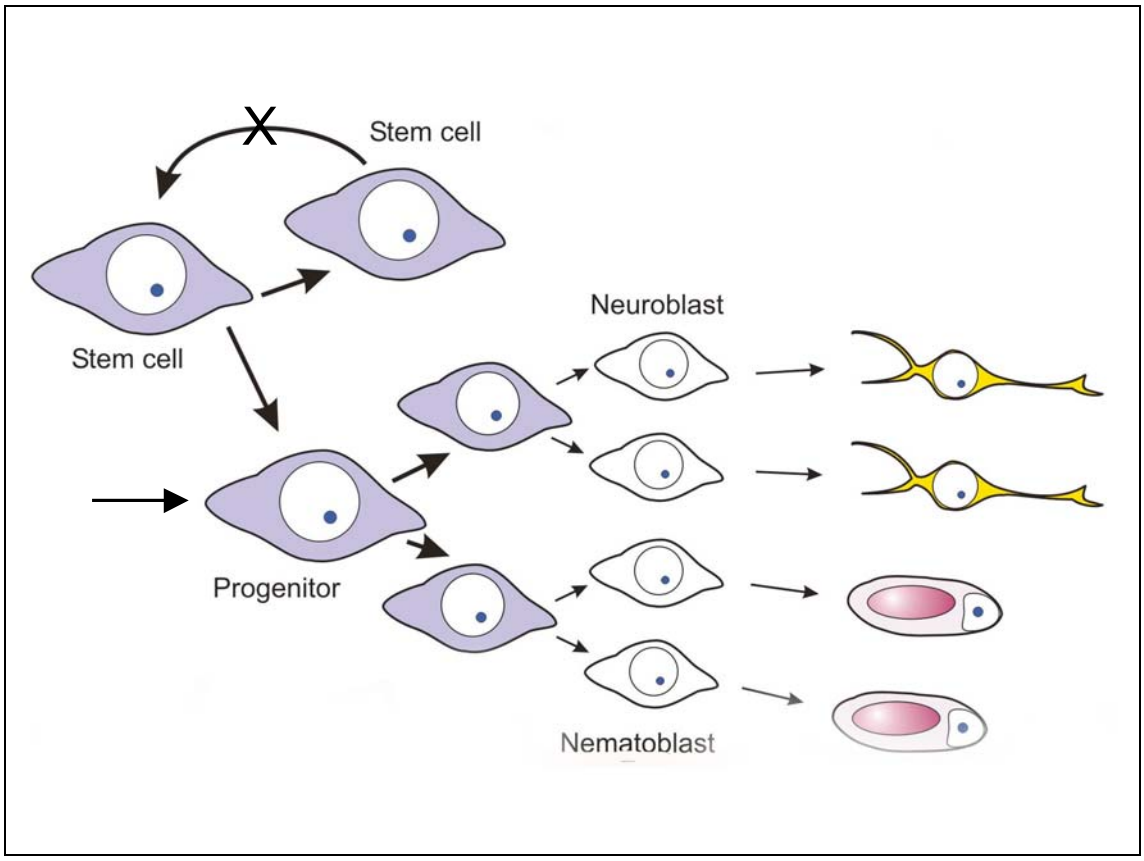

Figure 19. Model of the role WNT signalling plays in the stem cell fate of the subpopulation of nerve and stinging cells in *Hydractinia*, where WNT acts at the level of committed progenitors (arrowed) and not at the level of the basic stem cell population (crossed).

In contrast, with a long-term almost daily exposure of GSK-3β to alsterpaullone, the number of oocytes decreased and a large number of small I-cell like structures were observed. This lack of oocyte formation was also observed in a study on *Hydra* (Rentzsch et al., 2005). When the authors exposed the animals to daily exposure of inhibitors of GSK-3β, namely alsterpaullone or lithium, oocyte development did not proceed. Furthermore, *in situ* hybridisations with treated *Hydra* with the probe *HyGSK3* recognising transcripts of the *Hydra* GSK3β, gave a persistent upregulation of this transcript in the egg-restricted interstitial cells. *HyGSK3* transcription in untreated animals was downregulated when the oocyte had been selected and these egg-restricted I-cells embarked upon nurse cells and oocyte fates.

I propose that a similar event is occurring in *Hydractinia*. Figure 18B indicated that the almost daily treatment with alsterpaullone resulted in the presence of many small I-cell like structures in the gonozooids, with few or lacking any oocytes. However unlike what was proposed for Figure 19 for a pulse "WNT" signal, in Figure 20, I propose that the continuous signal received via alsterpaullone inhibition of GSK-3β, sustained the subpopulation of germ-restricted I-cells in the proliferative state, preventing its entry into differentiation.

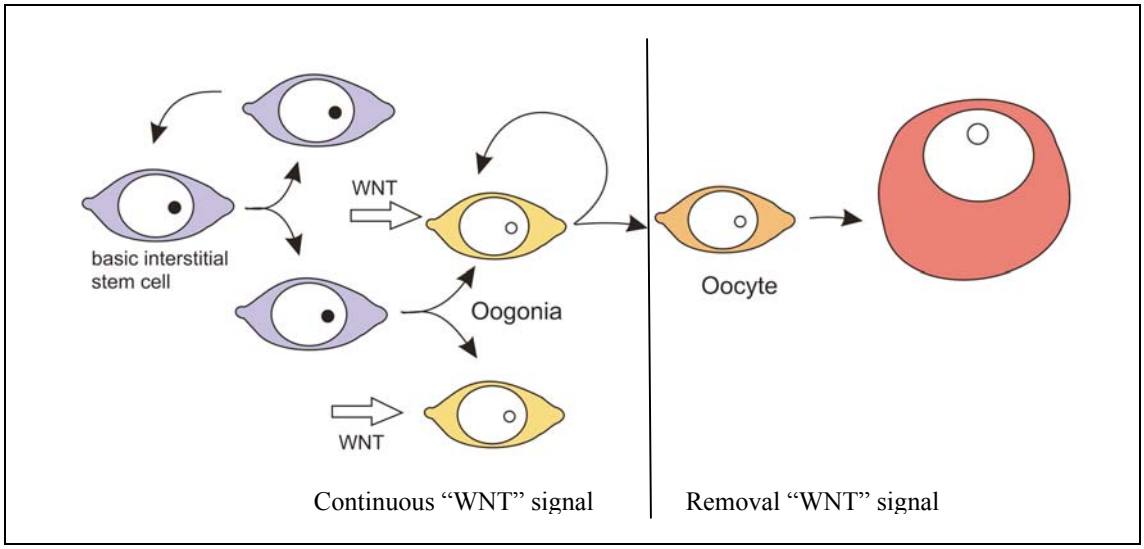

Figure 20. Model of the role WNT signalling plays in the stem cell fate of the subpopulation of germ cells in *Hydractinia*, where continuous WNT signal resulted in a self-renewal of the subpopulation of these oogonia.

Therefore, exposure of a WNT signal in both scenarios, will lead to a proliferation of the subpopulations of I-cells. However, it is only in the removal of the WNT signal that will eventually lead to a differentiation of these products.

The use of microsatellites in studying the fate of chimeric partners in *Hydractinia*

5.1 Allorecognition in *Hydractinia*

Hydractinia is a sessile marine colonial animal that grows asexually by lateral extension of its stolons. When these stolons come into tissue to tissue contact with isogeneic tissue, fusion results and subsequently a common, functional gastrovascular canal forms. Contact with allogeneic tissue results either in fusion or rejection brought about by a variation of effector mechanisms, or a combination of both events (review Frank et al., 2001, Fuchs et al., 2002, Cadavid et al., 2004 and references therein). Hence *Hydractinia* has histocompatible reactions in order to discriminate between self and non-self tissue.

Hauenschild (1954, 1956) first suggested that the transmission genetics of allorecognition in *Hydractinia* was attributed to a single locus co-dominant system. This hypothesis was expanded by Mokady and Buss (1996) using inbred lines where the authors showed that fusibility in *Hydractinia* was inherited in a Mendelian fashion, and attributed to a one locus system with co-dominant expression of the alleles. Sharing both alleles, or sharing at least one dominantly expressed allele, would allow two clones to fuse, forming a chimera, while non-sharing of any allele would result in no fusion but instead evoke mechanisms of mutual rejection. Recently Cadavid and coworkers (2004) showed that a linked two-loci model more precisely describes the genetics of allorecognition in *Hydractinia*. There appears to be a dose-dependent interaction of these two loci in controlling fusibility. Permanent fusion occurs when colonies share one or more alleles at both loci, rejection occurs if colonies share no alleles at either locus, and transitory fusion occurs if colonies share an allele at only one of the two loci.

This complexity of responses has also been documented by Fuchs and co-workers (2002). In their study on the ontogeny of the allorecogntion system, they produced chimeric embryos by combining two blastomeres, each of which taken from a different two-cell stage embryo, while the second blastomere from each of the two donor embryos were allowed to develop independently to naïve larvae (naïve here meaning not to have been in any physical contact to allogeneic cells). These naïve (half-sized larvae) gave rise to colonies that could be used to check the genetically programmed compatibility or incompatibility of the two combined halves of the

chimeric embryos. Most chimerical embryos did not separate during embryogenesis and developed into single larvae even when they were composed of two different compatibility histotypes. However, during or after metamorphosis, the tissues of such chimeras separated and developed into two incompatible primary polyps, and each of the two polyps turned out to be compatible to only one of the donors but incompatible to the other. If chimerical colonies separated only days or weeks after metamorphosis, transplantation assays between these separated colonies and the naïve founder colonies suggested that both incompatible genotypes were still represented in the formerly unified colonies. These results indicate that allorecognition markers and/or mechanisms of rejection develop fully only during or shortly after metamorphosis and that even allogeneic incompatible cells might survive after fusion.

Ecologically, this ability to recognise histocompatible types and form chimeras, may benefit one or both members of the chimera by 1. increased colony size, resulting in decreased total mortality since survivorship is known to be size dependent, 2. increased size, leading to a reduced age when the age of first reproduction begins and 3. having shared physiological attributes leading to increased tolerance to environmental variation (Rinkevich and Weissman 1992).

However fusion, permanent or transitory, results in the formation of a common gastrovascular system between the colonies, and the exchange of materials and particularly I-cells within the chimera. This also implies that though the animal phenotypically resembles one animal, it is in essence a chimera, habouring cells from both animals.

Potential benefits of chimera formation are counteracted by a great risk. Throughout the animal realm, precursors of gametes have migratory abilities. In *Hydractinia*, the migratory totipotent I-cells are also the precursors of gametes. Since histocompatibility is independent of sex, chimeras could be established between female and male colonies, and in these chimeras the female partner regularly lost their ability to produce eggs because highly proliferating spermatogonia derived from immigrated male precursors enumerated and displaced the oogonia in the goonozooids (Müller 1964). Eventually, also the original female partner of the chimera produced sperm from the immigrated, parasitic male precursors, increasing the fitness of the dominating clone at the expense of its own fitness. Similarly, in chimeras of urochordates such as *Botryllus schlosseri*, precursors of the gametes can compete for access to developing gonads (Pancer et al., 1995; Stoner et al., 1999).

Prompted by such observations, the hypothesis has been proposed that in evolution, histocompatibility arose in sessile invertebrates to prevent germ cell parasitism. High polymorphism of the allorecognition locus would ensure that histocompatibility is restricted to very close kin, where competition between different alleles is greatly reduced (Buss 1982, 1999, Rinkevich 1998; Magor et al., 1999).

5.2 The developmental potencies of the interstitial stem cells

The incentive for the present search for microsatellites came from studies with such chimeras. In order to examine the developmental potencies of the I-cells, Müller and co-workers (2004a) eliminated I-cells from a wild-type colony and introduced I-cells from a different, histocompatible, mutant clone. Donor and recipient were closely related (F_2 siblings or parent and child) but differed in several phenotypic traits. For instance they differed in their sex and the mutant had multiheaded polyps or raised stolons that did not adhere to the substrate. When a wild-type colony was depleted of I-cells and introduced with I-cells of the mutant donor, the recipient colony recovered, acquired the growth pattern of the mutant donor and with time even took on the sex of the donor. However, it has also been observed that one out of the 12 chimeras established with the same recipient and donor clones, returned to the phenotype and sex of the wild-type recipient colony (Müller pers. comm.). This indicated long-term survival of some I-cells from the wild-type clone in the chimera. Furthermore, given the totipotent ability of the I-cells of *Hydractinia* (Müller et al., 2004a), in transitory chimeras, circulating donor I-cells may manifest themselves phenotypically and at the level of the germ cells only much later after the fusion-separation event. Genotyping of the chimeras at somatic and germ line level would therefore help to assess the advantage or disadvantage of such forms of parasitism and these could be performed via the use of molecular markers.

5.3 Molecular markers of individual identity

Individual organisms differ in their DNA sequences and this variation at the gene level may be expressed phenotypically or be recognisable only at the genotype level with the use of neutral molecular markers. With the advance in the tools of molecular biology, many molecular markers have been developed for their use in questions related to population genetics and evolution of genes (e.g., microsatellites linked to loci of Major Histocompatibility Complex).

Several molecular markers were developed to serve as a source of neutral markers in answering questions related to organismal relationships (Avise 1993). This neutrality meant that these variations do not show up in the phenotype and do not disturb the physiology of the animal but are however more numerous than morphological markers (Jones et al., 1997). Currently, large numbers of molecular markers have already been developed (see Table 4, source:http://www.cgn.wageningen-ur.nl/pgr/research/molgen/) and their usage depends on the how fine the genetic discrimination is required for the question being addressed and the rate of evolution of this marker.

Table 4. List of molecular markers generally in use for population studies and its efficiency of usage. Taken from http://www.cgn.wageningen-ur.nl/pgr/research/molgen/. A similar table is given in a review in Sunnucks (2000). RFLP = Restriction Fragment Length Polymorphism. RAPD = Random Amplified Polymorphic DNA. ISSR = Inter Simple Sequence Repeats. SSCP = Single-Strand Conformation Polymorphism. CAPS = Cleaved Amplified Polymorphic Sequences. SCAR = Sequence Characterized Amplified Region. AFLP = Amplified Fragment Length Polymorphism.

	Allozymes	RFLP	Minisatellites	PCR sequencing	RAPD	Microsatellites
abundance	Low	High	medium	Low	High	High
level of polymorphism	Low	medium	High	Low	Medium	High
locus specificity	Yes	Yes	No/yes	Yes	No	Yes
codominance of alleles	Yes	Yes	No/yes	Yes	No	Yes
reproducibility	High	High	High	High	Low	High
labor-intensity	low	High	High	high	Low	Low

(Table continued)

	ISSR	SSCP	CAPS	SCAR	AFLP	TaqMan
abundance	Medium-high	Low	Low	Low	High	low
level of polymorphism	Medium	Low	Low-medium	Medium	Medium	
locus specificity	No	Yes	Yes	Yes	No	yes
codominance of alleles	no	yes	yes	Yes/no	No/yes	No/yes
reproducibility	Medium-high	medium	High	High	High	High
labor-intensity	low	Low-medium	Low-medium	low	medium	low

Table 4 indicates that the level of polymorphism is high in microsatellites, suggesting that the rate of mutation is proportionately high (review, Ellegren 2004) and therefore this polymorphism of microsatellites can serve as a powerful tool for analysing recent genetic variations (review, Wan et al., 2004). Furthermore, with their high reproducibility, locus specificity and the inheritance of alleles in a co-dominant Mendelian manner, microsatellites are suitable in addressing issues as discrimination, relationships and classification within the population, but also to identify individuals (Wan et al., 2004). All these factors support the use of microsatellites as molecular markers of choice in following the fate of chimeric partners in *Hydractinia*.

5.4 Microsatellites

Microsatellites are a class of short tandemly repeated DNA sequences comprising 1-6 base pairs (bp) per repeat unit. They are usually 6-30 repeat units long. Due to their versatile applicability from forensics and diagnosis of diseases, to population and conservation biology, microsatellites were used as markers in very different contexts and are also known in the literature as simple sequence repeats (SSR), short tandem repeats (STR) or variable number tandem repeats (VNTR). However, microsatellites are more than simple, perfect repeats. Within their classification, microsatellites comprise also compound repeats (tracts of different repeats), interrupted repeats (inserted base interrupting the repeat) and imperfect repeats (mutation of one base in the repeat motif to another base).

Microsatellites in humans are located both in coding and non-coding regions, however the bulk of them are in introns. Those within coding regions cause mutations that can serve as hereditary markers in cancer studies (Sturzeneker et. al, 2000) or in human diseases, e.g., Huntington's chorea (Leeflang et. al., 1999), while those within non-coding regions can be used as universal genetic markers as they are generally assumed to evolve neutrally (see review, Ellegren 2004).

Experimental studies for human microsatellite mutations *in vivo* showed that mutations occurred at rates of 10^{-4} to 10^{-2} per locus per generation (Lai and Sun 2003). It is generally accepted that the high polymorphism observed in microsatellite loci is attributed to this quick rate of evolution (or mutation). Pedigree analyses (references in Ellegren 2004) support the theory that the mechanism by which microsatellites mutate is by slip strand mispairing (or replication slippage) of which a slippage mutation threshold size exists (Lai and Sun 2003) for mutation to occur.

Concurrent with experimental results, two sets of equations based on two stochastic processes to study estimate slippage mutation rates proposed by Lai and Sun (2003) indicated that small number of repeat units tends to expansion and long microsatellites tend to contract. Furthermore, this rate of substitution appears to be higher at the very end of microsatellites than in the middle of repeat regions (Brohede and Ellegren 1999).

In contrast to mutations that lead to high polymorphism, stability results in otherwise. Two factors influencing microsatellite stability are repeat counts and purity. In *Drosophila*, mutation rates increase with microsatellite loci that had higher repeats counts (Goldstein and Clark 1995), a similar trend was found also in perfect $(CA)_n$ repeats of humans (Weber 1990). Weber also found that long runs of perfect repeats are good predictors of polymorphism. Interrupting bases however, tend to stabilise repeat tracts, leading to lower rates of mutations, and subsequently also lower rates of polymorphism (Pépin et. al., 1995). Therefore this phenomenon probably explains why polymorphism at the same locus among species/populations can also be different. Currently there is no perfect mutation model to explain the mutation process (stepwise mutation model or otherwise) due to heterogeneous data with regards to specific loci, repeat types and species (Ellegren 2004).

In general it is the high degree of polymorphism assumed in microsatellites that makes them attractive as markers. Using human loci representing di-, tri- and tetranucleotide repeats and mathematical mutation models, Chakraborty and coworkers (1997), found that loci mutation rates were inversely proportional to the size of the repeat unit. Hence, dinucleotide loci appear to be evolving at a rate 1.5-2.0 times faster than tetranucleotides. Composition of repeat units appears to also be a factor for microsatellite polymorphism (Estoup and Cornuet quoted in Goldstein and Schlötterer, 1999). AT-rich tracts appear to be more polymorphic than those with GC-tracts, which tend to stabilise the repeat units. Population size is also a factor in the degree of polymorphism observed. Studies (Whitehouse et. al., 2001, Gaustchi et. al., 2003) have shown that polymorphisms were fewer in small endangered populations and loss of alleles over generations was significant in small populations, with 87.5% of endangered animals having less than 5 alleles per locus on average compared to 19.2 in non-endangered animals.

Mutation at flanking regions of repeats also appears to be a common phenomenon. Although substitution in flanking regions of repeats appears to be no

different from that of intron sequences and hence appears to be selectively neutral, in a study conducted by Brohede and Ellegren (1999) on twenty-two mammalian $(CA)_n$ repeats, they found that the substitution rate was significantly higher within the first five nucleotides flanking the repeat than in sequences further away. Furthermore, in a study on eight primate loci using human primers, insertion/deletion (indel) events comprising 1-5 nucleotides were found to also occur at high frequency (Clisson et al., 2000).

All the above factors, together with the possibility of point mutations within the flanking regions, can lead to the phenomenon of null alleles. Null alleles are non-functional alleles and frequently identified because of reduced or loss of polymerase chain reaction (PCR) products when microsatellite primers fail to locate the complementary sequence for annealing. Consequently, there appear to be fewer heterozygotes within a population than expected. Taken together, although the use of microsatellites in population studies appears attractive, the intrinsic problems involved in locating suitable polymorphic loci within a species and subsequently within the population, have to be first overcome.

Once potentially useful microsatellites are found and PCR primers constructed from the DNA sequences flanking the microsatellite regions amplify polymorphic loci, these PCR-amplified microsatellite alleles can be detected using various methods e.g. silver staining. PCR-based microsatellite analysis has also the advantage of requiring small amounts of DNA for the reaction and giving results in 48 hours or less. Furthermore the results are usually reproducible. Finally, microsatellite alleles have discrete sizes, allowing simplified interpretation of results.

5.5 Results

5.5.1 Screening for presence of suitable repeats

A PCR on the genomic DNA was first conducted with primers representing different repeat types to ascertain what sorts of repeats were present in *Hydractinia*. A list of the primers used and the repeat-type present in *Hydractinia* is given in Table 5. The sample of primers tested indicates that *Hydractinia* has repeat types from di-, tri- and tetranucleotides but the trinucleotide repeat (TCC), the pentanucleotide repeat (GACAC) and the compound repeat $(CT)_4(CA)_5$ were not present (Fig. 21).

Table 5. List of primers used to test for presence of repeats in *Hydractinia*. Names given correspond to gel picture in Figure 21.

Name of repeat	Type of repeat	Present
A	$(CA)_{10}$	*
B	$(CT)_{10}$	*
C	$(GA)_{10}$	*
D	$(GT)_{10}$	*
E	$(CAG)_5$	*
F	$(GTG)_5$	*
G	$(TCC)_5$	No
H	$(CT)_4(CA)_5$	No
I	$(GGA)_4$	*
J	$(GGAT)_4$	*
K	$(GATA)_4$	*
L	$(GACA)_4$	*
N	$(ACGA)_4$	*
O	$(TGTC)_4$	*
P	$(GACAC)_3$	No
Q	$(ACTG)_4$	*

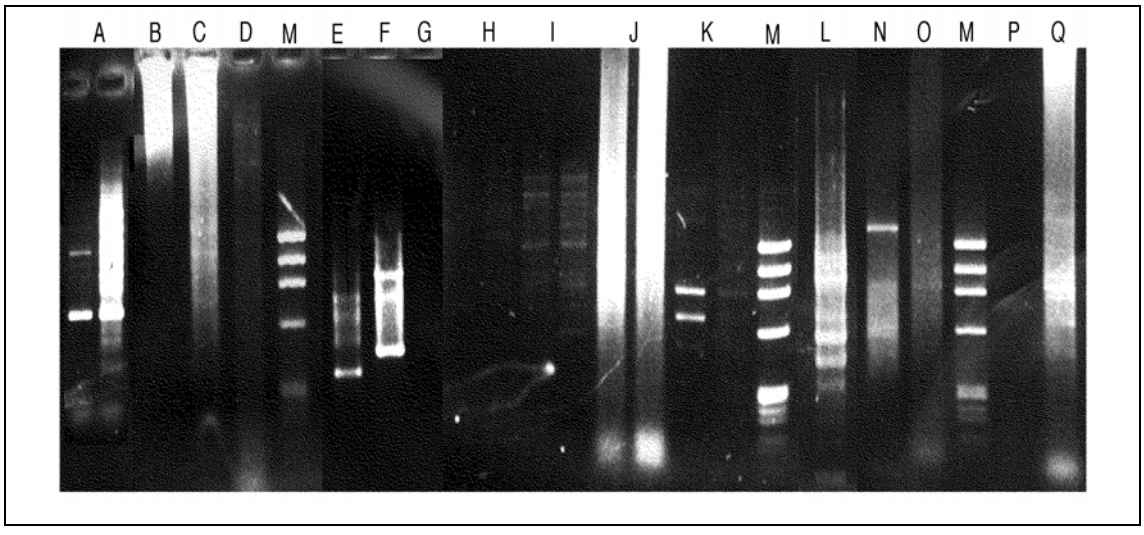

Figure 21. Screening for repeats within *Hydractinia*. Alphabets correspond to repeat type found in Table 5. M = Biosizer marker, with highest band corresponding to 1353 bp, with fourth and fifth bands from top corresponding to 603 bp and 310 bp respectively. Alphabets spanning two gel slots were replicate PCR with the same repeat-primer.

Figure 21 indicates the probable quantity and size ranges of repeats within the *Hydractinia* genome. All screenings were carried out with the same conditions, except for differences in annealing temperature depending on the repeat type. The intensely stained smears obtained for repeats $(CT)_{10}$, $(GA)_{10}$, $(GGAT)_4$ and $(ACTG)_4$ suggest high quantities of these repeats and wide ranging sizes. The dinucleotides $(CA)_{10}$, and trinucleotides $(CAG)_5$, $(GTG)_5$ appear to have smaller ranges of repeat sizes with sufficient quantities.

Many human diseases are known to be associated with the expansion of tracts of (CAG)-repeats. Since such repeats may be within gene coding areas not only in humans but also in *Hydractinia*, and may therefore be not neutral, screening for (CAG)-repeats was not carried out. Establishment of libraries was carried out for the repeat types $(CA)_{10}$ and $(GTG)_5$. Colony lift hybridisations for $(GTG)_5$ gave no positive clones. Screening for suitable microsatellites loci was hence carried out with the library made for the repeat type $(CA)_{10}$.

A total of 213 clones were identified as (CA)-positive from the colony lift hybridisations. A second round of screening that was carried out with the dot blot hybridisations of the plasmid DNA gave negative signals for 35 (16.4%) out of the 213 clones i.e., no (CA) tracts were found. A total of 107 (60.1%) clones from the remaining (178) were sequenced, of these 17 (15.9%) contained no microsatellites. Of the remaining 90 found to have microsatellites, a total of 31 sequences had perfect repeats with no substitutions and 59 sequences had substitutions (imperfect repeats) and/or interruptions in the repeats. Seven sets of sequences had repeat clones, with designation of repeat clones as follows: (2G, 2L), (2I, 2J), (8, 9), (22, 28), (48, 57, 67), (81, 91), (96, 97, 98, 99).

To decrease incidences of false typing of alleles due to slip strand mispairing during the PCR reaction, a maximum product size of approximately 300 bp was chosen as the limit for selecting appropriate loci for polymorphism testing. Furthermore, only those loci with flanking sequences that would give primer sequences that were potentially located at least eight base pairs away from the repeats, were selected. Using these criteria, a total of 8 loci from the list of perfect repeats, and 25 loci from the imperfect microsatellites were deemed appropriate for screening. This translated to 36.6% of the number of microsatellites sequenced. A total of 19 loci from both repeat sets were tested for polymorphism. Lists of primer sequences used are given in the Materials and Methods and the loci tested in Tables 6-9.

A total of 8 loci tested gave null alleles and 7 gave multiple bands when checked on the agarose gels. Of the remaining 4 loci, #48, #66, #81, #133 gave either single or double bands and were further tested for polymorphism either on polyacrylamide gels or via sequencing. Locus #133 was later abandoned when further PCRs showed inconsistencies in the products obtained. Details of these results are given in Tables 6-9.

5.5.2 Testing for polymorphism in chimeras

In the study conducted by Müller and co-workers (2004a), it was shown that histocompatible combinations between a wild type and a mutant colony resulted in the colony eventually being phenotypically represented in the entire colony when I-cells were not depleted from either colony before the fusion process. On the other hand, when I-cells were completely depleted from one colony, and new I-cells introduced from a histocompatible donor, the I-cell depleted colony recovered and with time even took over the phenotype of the donor, down to the sex cells. Genotypically, the question as to whether this colony was still a chimera or has converted completely into donor can only be answered with the use of suitable molecular markers. And this was attempted with the use of locus 81 on the naïve colonies of Male 1 (Ma) and female 7x7 –14 (hence abbreviated 7x7) the daughter from the F_1 cross of male # 7 and female # 7, who were both siblings of Ma, and their compatible fusions. The chimeras A-E, L-M had the phenotype of 7x7 although such chimeras showed phenotypically Ma traits if the I-cells of Ma were not eliminated prior to fusion.

PCR conducted on the genomic DNA of the naïve colonies of Ma and 7x7, and seven chimeras showed the presence of a single band on the agarose gel (Fig. 22). To check the degree of polymorphism, these same PCR products were ran on a denaturing polyacrylamide gel (Fig. 23). Results here suggested that Ma and 7x7 were different from each other and that the putative chimeras eventually consisted genotypically of 7x7 cells only.

Table 6. List of microsatellites with perfect stretch of repeats but with no suitable flanking regions for primer design. Table 6 to Table 9 give a summary of the types of repeats found in *Hydractinia* via sequencing and the length of the flanking regions. usat = microsatellite. Left/Right flanking = stretch of sequence in bp that was readable, up till the restriction site corresponding to the ligation site into the plasmid. ? = sequence stretch was ambiguous, such that correct determination of number of bases was not possible. Names in bold indicate these loci were tested for polymorphism. All repeat types shown here were presented as observed in the sequence when sequencing reaction was carried out with the T_3 polymerase of the plasmid. tg = thymidine-guanine repeats; ca = cytosine-adenosine repeats.

Name of usat	Length of usat	Perfect	Compound	Left flanking	Right flanking
96	?	tg		95	?
97	?	tg		95	?
98	?	tg		95	?
99	?	tg		95	?
34	60	tg		20	?
44	>20	tg		45	?
46	>20	tg		86	?
47	>30	tg		9	?
49	>104	tg		9	?
70	>20	tg		98	?
105	>20	tg-ca	*	25	?
21	>20	tg-tg	*	50	?
24	>20	tg		>90	?
26	>60	tg		38	?
27	>170	ctaca		52	?
31	>70	ca		44	?

7	?	caa		4	?
1D	>30	tg-ca	*	444	?

Table 7. List of microsatellites with perfect stretch of repeats with suitable flanking regions for primer design. Microsatellite names listed according to length of repeat tract.

Name of usat	Length of usat	Perfect	Left flanking	Right flanking	Primers designed	Remarks
133	130	ca	128	28	Y	
4	30	ca	55	167	N	
6	84	ca	52	46	N	
8	80	ca	120	>90	N	8,9 same
9	81	ca	121	>91	N	8,9 same
48	58	ca	120	>100	Y	48,57,67 same; not polymorphic
57	56	ca	121	>101	N	48,57,67 same; not polymorphic
67	58	ca	116	>110	Y	48,57,67 same; not polymorphic

Table 8. List of microsatellites without suitable flanking regions for primer design. Sequence is given in order as observed. Single base indicate the type of substitution. Stretches of 4 or more base pairs indicate interruption in the repeat tracts. Microsatellite names listed according to length of repeat tract.

Name of usat	Length of usat	Repeat type	Imperfect	Interrupted	Compound	Left flanking	Right flanking
1A	>500	ca-t	*			6	?
12	>430	ca-t	*			46	?
29	>400	ca-t	*			17	?
71	>400	ca-t-g	*			30	?

102	258	ca-t-g-ccc	*	*		42	?
91	108	ca-cat				66	?
51	>100	tg-c	*			100	?
92	70	ca-ct			*	?	?
167	>70	gtt-gt-a-c-aa	*		*	97	?
110	300	ca-t	*			7	25
80	280	ca-cata			*	14	8
19	187	ca-aag		*		26	>100
2J	181	ca-tgggtcaagtaa-g	*	*		4	86
2I	180	ca-tgggtcaagtaa-g	*	*		4	85
43	170	ca-g-t	*			24	45
1L	140	ca-t-cata-ca		*	*	14	101
2K	110	ca-g-t-ccc	*	*		4	75
108	100	ca-cg-t-tgctt-ca-g-a		*	*	4	>90
2L	338	ca-t-a-g	*			42	9
89	306	ta-t-ca-g-catg-tatg-tcgt-ca-t-g-a	*	*	*	45	4
2G	286	ca-t-a-g	*			40	9

38	270	ca-t-g-cccg	*	*		45	25
55	220	ca-cata			*	68	26
32	200	ca-t	*			37	17
72	148	ca-ggcc		*		35	4
60	140	tg-ttgc-aagca-tg-cg-ca-tt-gc-tg-c	*	*	*	170	6
1E	>120	tg-ca-t-g-aaa	*	*	*	340	20
5	110	ca-cat			*	68	29
25	90	ca-t-aaaa	*	*		82	>20
1J	48	tg-tttg			*	254	28

Table 9. List of microsatellites with suitable flanking regions for primer design. Sequence is given in order as observed. Single base indicate substitution within the repeat stretch. Sequences of 4 or more base pairs indicate interruption in the repeat tracts. Only those in bold were tested. Microsatellite names listed according to length of repeat tract.

Name of usat	Length of usat	Repeat Type	Imperfect	Interrupted	Compound	Left flanking	Right flanking
2F	>400	ct-ca-t	*		*	70	66
93	380	ca-t-g-a				39	30
62	370	ca-t				40	30
1G	345	ca-ccat		*		43	30
179	324	ca-t-g	*			51	37
3	320	ca-g-t	*			44	53

94	300	ca-t-g	*			62	38
1	290	ca-g-t	*			41	86
95	280	ca-t-g	*			55	50
35	270	ca-t-g-gcaagc	*	*		45	34
1B	260	ca-g-t	*			60	72
2C	>250	ca-t-c-g	*			44	111
30	250	ca-ggcc		*		44	46
1H	220	ca-gg		*		34	50
2P	206	cacaa-ca-g	*		*	118	40
41	200	ca-cata-ca-t-caaa	*	*	*	60	40
2B	182	ca-cca-t	*		*	89	80
128	180	ca-a	*			41	>150
2H	162	ca-g-t	*			44	64
111	154	ca-t-g-a-caaa	*	*		46	54
88	150	ca-t-g	*			107	80
52	150	ca-ta-ctcatt-ca-cgcatt-g	*	*	*	31	85
58	138	ca-g-t-aa	*	*		40	62
66	130	ca-gcaa		*		38	54
73	116	c-ca-t-g	*		*	46	>100

5	110	ca-cat			*	68	29
59	110	ca-t-g	*			44	43
1K	110	ca-t-g	*			40	40
104	90	ca-g-cacaactt	*	*		81	>50
81	72	ca-cata-g-aaaa	*	*		81	>70

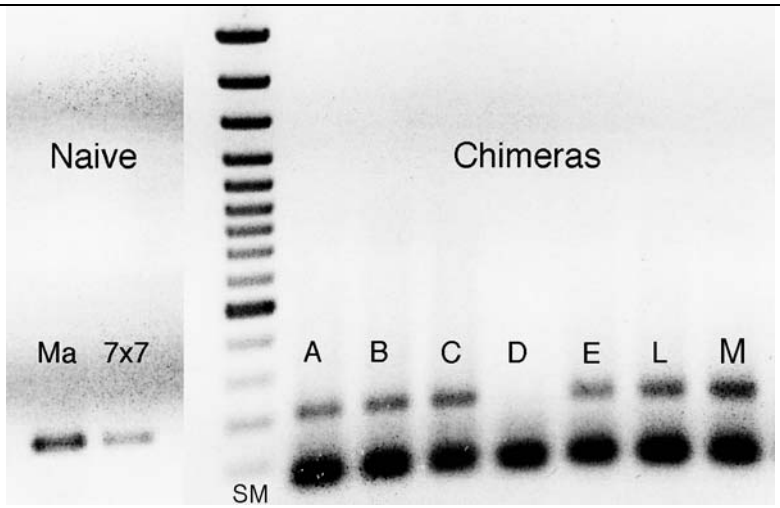

Figure 22. Agarose visualisation showed presence of single band obtained for locus 81 on naïve and chimeric colonies. Ma = Male1, 7x7 = mutant F_2, A-E, L-M = Chimeras of Ma and 7x7. PCR on chimera D gave no result. SM = size marker, Gene Ruler 100 bp Plus with first five bands from bottom each representing 100 bp difference. The highly intense fifth band represents a size of 500 bp.

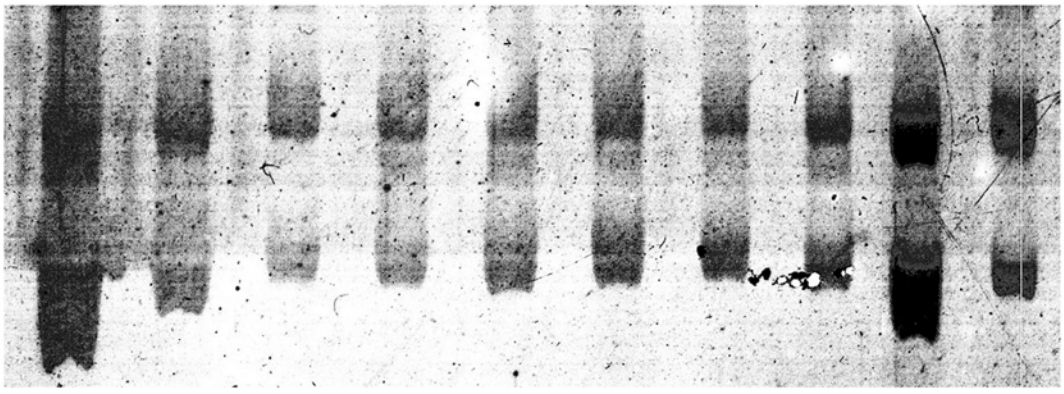

Figure 23. Screening for polymorphism on denaturing polyacrylamide gel. Only smallest two bands represented.

Direct PCR of the denaturing gel products was carried out on the four bands of Ma and 7x7 to determine how many repeat unit differences was represented by these bands. The PCR products of these were subsequently cloned and sequenced. PCR products of the chimeras shown in Figure 22 were also cloned and sequenced. An alignment of the sequences obtained is given in Figure 24. For the full sequences obtained, the alignment indicates high stability of this locus before the start of the CA-repeat type. Sequences MaUp and 7x7 indicate the absence of one CA-repeat at the border between the repeat type and the flanking regions.

CLUSTAL W (1.82) multiple sequence alignment

```
Ma          ----------GGTGTGTACGCTGATACACTTATGCATTGGTACATTGAAACATTGAAAC      49
MaUp        ----------GGTGTGTACGCTGATACACTTATGCATTGGTACATTGAAACATTGAAAC      49
MaLow       ----------GGTGTGTACGCTGATACACTTATGCATTGGTACATTGAAACATTGAAAC      49
7x7         ----------GGTGTGTACGCTGATACACTTATGCATTGGTACATTGAAACATTGAAAC      49
7x7Up1      ----------GGTGTGTACGCTGATACACTTATGCATTGGTACATTGAAACATTGAAAC      49
7x7Up2      ----------GGTGTGTACGCTGATACACTTATGCATTGGTACATTGAAACATTGAAAC      49
7x7Low      ----------GGTGTGTACGCTGATACACTTATGCATTGGTACATTGAAACATTGAAAC      49
ChimeraA    ----------GGTGTGTACGCTGATACACTTATGCATTGGTACATTGAAACATTGAAAC      49
ChimeraB    ------------------------------------------------------------
ChimeraC    ----------GGTGTGTACGCTGATACACTTATGCATTGGTACATTGAAACATTGAAAC      49
ChimeraE    ----------GGTGTGTACGCTGATACACTTATGCATTGGTACATTGAAACATTGAAAC      49
ChimeraL    ------------------------------------------------------------
ChimeraM    ----------GGTGTGTACGCTGATACACTTATGCATTGGTACATTGAAACATTGAAAC      49

Ma          ACATGTACATACATGCACACATACACACATACACACAAAACACACACACACATACACACA     109
MaUp        ACATGTACATACATGCACACATACACACATACACACAAAACACACACACACATACACACA     109
MaLow       ACATGTACATACATGCACACATACACACATACACACAAAACACACACACACATACACACA     109
7x7         ACATGTACATACATGCACACATACACACATACGCACAAAACACACACACACATACACACA     109
7x7Up1      ACATGTACATACATGCACACATACACACATACACACAAAACACACACACACATACACACA     109
7x7Up2      ACATGTACATACATGCACACATACACACATACACACAAAACACACACACACATACACACA     109
7x7Low      ACATGTACATACATGCACACCTACACACATACACACAAAACACACACACACATACACACA     109
ChimeraA    ACATGTACATACATGCACACATACACACATACACACAAAACACACACACACATACACACA     109
ChimeraB    ----------------------------------CAAAACACACACACACATACACACA      25
ChimeraC    ACATGTACATACATGCACACATACACACATACACACAAAACACACACACACATACACACA     109
ChimeraE    ACATGTACATACATGCACACATACACACATACACACAAAACACACACATACATACACACA     109
ChimeraL    ----------------------------------CAAAACACACACACACATACACACA      25
ChimeraM    ACATGTACATACATGCACACCTACACACATACACACAAAACACACACACACATACACACA     109
                                              ************ ***********

Ma          TACATACACACACACACACACACGCACAGGGGGGTACCAGGAATGATGTTGGAAGAGGA      167
MaUp        TACATACACACACACACACAC--GCACAGGGGGGTACCAGGAATGATGTTGGAAGAGGA      165
MaLow       TACATACACACACACACACACGCACAGGGGGGTACCAGGAATGATGTTGGAAGAGGA      167
7x7         TACATACACACACACACACAC--GCACAGGGGGGTACCAGGAATGATGTTGGAAGAGGA      165
7x7Up1      TACATACACACACACACACACGCACAGGGGGGTACCAGGAATGATGTTGGAAGAGGA      167
7x7Up2      TACATACACACACACACACACGCACAGGGGGGTACCAGGAATGATGTTGGAAGAGGA      167
7x7Low      TACATACACACACACACACGCGCACAGGGGGGTACCAGGAATGATGTTGGAAGAGGA      167
ChimeraA    TACATACACACACACACACGCGCACAGGGGGGTACCAGGAATGATGTTGGAAGAGGA      167
ChimeraB    TACATACACACACACACACACGCACAGGGGGGTACCAGGAATGATGTTGGAAGAGGA       83
ChimeraC    TACATACACACACACACACACGCACAGGGGGGTACCAGGAATGATGTTGGAAGAGGA      167
ChimeraE    TACATACACACACACACACACGCACAGGGGGGTACCAGGAATGATGTTGGAAGAGGA      167
ChimeraL    TACATACACACACACACACACGCACAGGGGGGTACCAGGAATGATGTTGGAAGAGGA       83
ChimeraM    TACATACGCACACACACACACGCACAGGGGGGTACCAGGAATGATGTTGGAAGAGGA      167
            ********************  ************************************

Ma          CGAGGCGATTTCCGGGTGAGACGAGAGGTCCAG------------------------------ 200
MaUp        CGAGGCGATTTCCGGGTGAGACGAGAGGTCCAG------------------------------ 198
MaLow       CGAGGCGATTTCCGGGTGAGACGAGAGGTCCAG------------------------------ 200
7x7         CGAGGCGATTTCCGGGTGAGACGAGAGGTCCAG------------------------------ 198
7x7Up1      CGAGGCGATTTCCGGGTGAGACGAGAGGTCCAG------------------------------ 200
7x7Up2      CGAGGCGATTTCCGGGTGAGACGAGAGGTCCAG------------------------------ 200
7x7Low      CGAGGCGATTTCCGGGTGAGACGAGAGGTCCAG------------------------------ 200
ChimeraA    CGAGGCGATTTCCGGGTGAGACGAGAGGTCCAG------------------------------ 200
ChimeraB    CGAGGCGATTTCCGGGTGAGACGAGAGGTCCAG------------------------------ 116
ChimeraC    CGAGGCGATTTCCGGGTGAGACGAGAGGTCCAG------------------------------ 200
ChimeraE    CGAGGCGATTTCCGGGTGAGACGAGAGGTCCAG------------------------------ 200
ChimeraL    CGAGGCGATTTCCGGGTGAGACGAGAGGTCCAG------------------------------ 116
ChimeraM    CGAGGCGATTTCCGGGTGAGACGAGAGGTCCAG------------------------------ 200
            ********************************
```

Figure 24. CLUSTALW alignment of the sequences indicates sequences lengths to do not correspond to what was observed on the gel as depicted in Figure 23. Up = top band observed and Low = bottom band observed in Figure 23. Numbers next to names indicate plasmid replicates sent for sequencing from the same PCR reaction. Bold numbers indicate inconsistency of sequence size with respect to position on gel picture Figure 23.

Furthermore, the sequences indicated that the sizes obtained do not correspond with that what was observed on Figure 23. For example, MaUp, which on Figure 23 represents the larger band in comparison to MaLow, had instead a size of 198 bp, and the other, a size of 200 bp. A further confirmation of the inconsistency of results was represented by 7x7Up1, 7x7Up2 and 7x7Low, which all gave the same sequence size of 200 bp, while according to Figure 23, 7x7Low should represent a smaller repeat.

To further check the results obtained in Figures 23-24, another denaturing polyacrylamide gel was carried out on Loci 48 and 81, representing product sizes 203 bp and 204 bp respectively, to see how these differences should be represented. The number of bands obtained (Fig. 25) suggested the unsuitability of this gel for purposes of the current study and the bands obtained in Figure 23 were probably stutter bands.

Detection for polymorphism was carried out again on the naïve and chimeric animals after optimising the gel conditions, most notably 1 mm thick gel instead of 0.4 mm thick. These results indicated that there were probably no polymorphisms observed for the naïve and chimeric colonies for locus 81 (Fig. 26) and that this gel was better at discriminating any possible polymorphism.

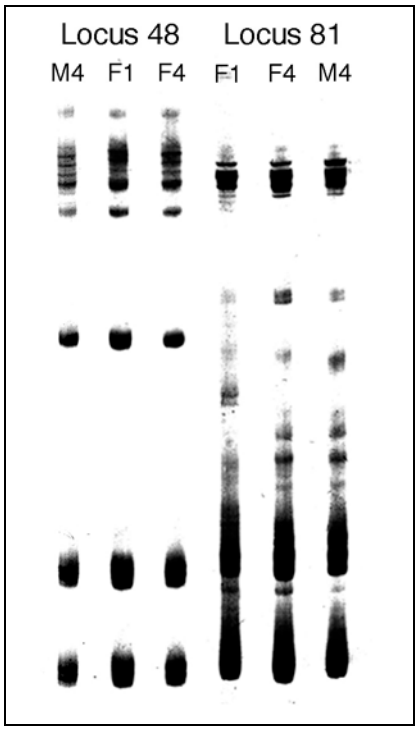

Figure 25. Comparison of loci 48 and 81 on three wild-type animals. Estimated product sizes of locus 48 and locus 81 are 203 and 204 respectively.

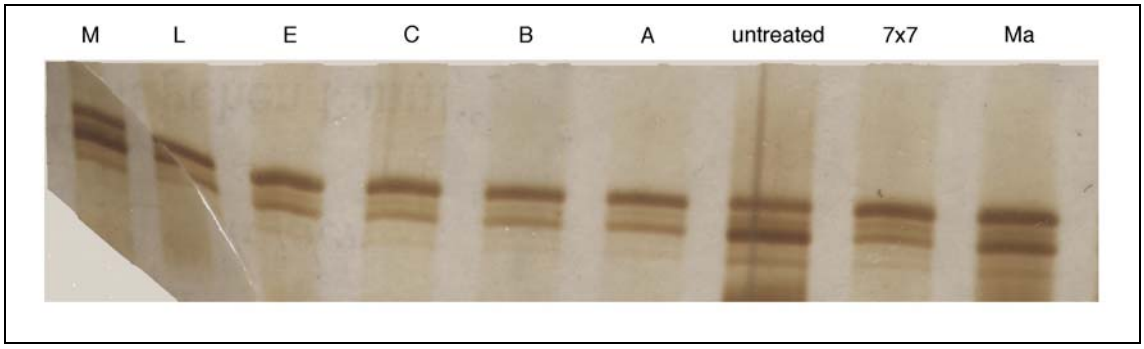

Figure 26. Replicate run of the products from Figure 23 on thick gel. “untreated” represents a colony which phenotypically resembles Ma, although it may be a chimera. Neither colony from this chimera was depleted of I-cells before the fusion process.

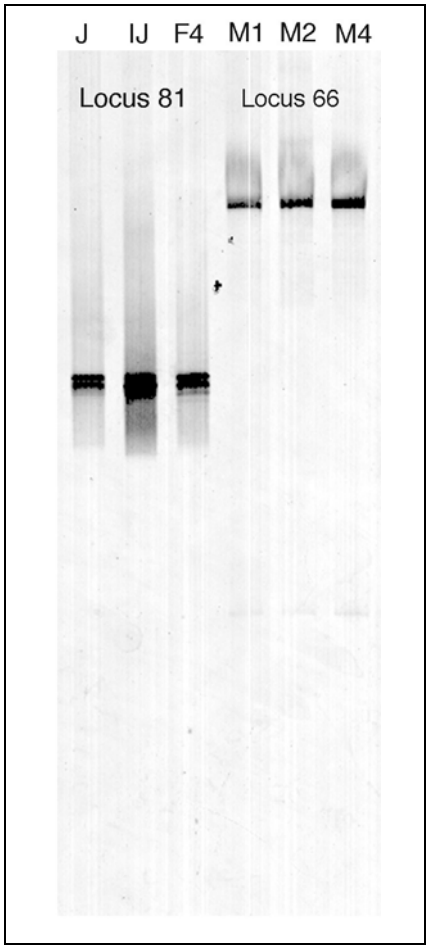

Figure 27. A denaturing polyacrylamide gel on locus 81 (204 bp) and locus 66 (226 bp) to ascertain the estimated visual length difference for approximately 20-22 bp.

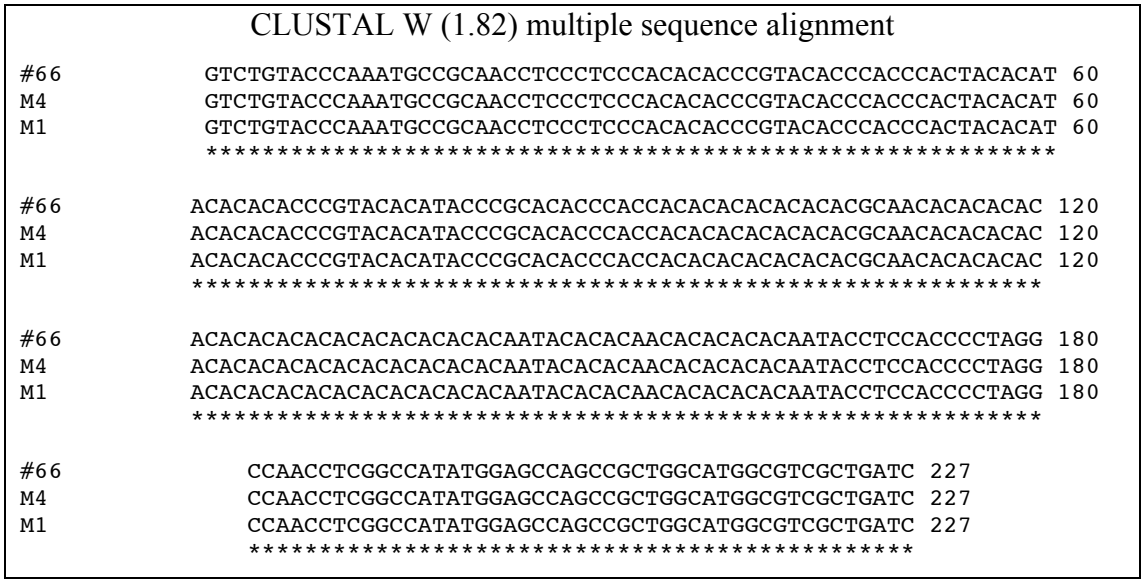

Figure 28. Sequence alignment of the Plasmid from locus 66 and two wild-type colonies, M1 and M4.

Finally, in order to ascertain the length differences that should be observed on this newly optimised gel, products from loci 81 and 66, representing 204 bp and 226 bp were separated on this gel (Figure 27). Initial estimation indicated that at least an 8 bp difference was necessary for a convincing indication of polymorphism. Sequence alignment of the clones M1 and M4 for locus 66 (Figure 28) indicated that results obtained showed no ambiguity in the sequences. Further screening for polymorphic loci continued.

5.6 Discussion and Conclusions

Many methods have been developed for isolation of microsatellites (review Zane et al., 2002). The method used for the current study was that of enrichment, involving 'fishing' of the appropriate repeat types with biotin-labelled, in this case biotin-$(CA)_{10}$ probes, and subsequently capturing these stretches of DNA with streptavidin beads. This method improved the efficiency of returned positive clones compared to traditional shotgun cloning methods which normally yield only 2-3% positive clones in relation to the total number of clones sequenced, and usually 50% of these positive clones had to be discarded due to lack of suitable flanking sequences for primer design or inconsistent PCR products. In the current study, of a small sample of 107 sequenced clones, 90 of these contained microsatellites, representing 84% of the pool, thereby highlighting the relative successful rate of this enrichment technique in isolating microsatellites in *Hydractinia*.

The presence of stutter or shadow bands observed in Figures 23 and 25 is a phenomenon that was already first documented in 1993 by Murray and co-workers (Murray et al., 1993). They proposed that these bands were the result of 2 base pair random deletions in the (CA)-repeat region since sequencing of these shadow bands showed ambiguity only in these regions and not in the flanking regions of the repeat tract. Later studies (Liepelt et al., 2001, Clarke et al., 2001) confirmed that generally during PCR, both Taq polymerase and proofreading polymerase Pfu caused loss of repeat units at similar error rates, thereby accounting for the series of different bands observed for a single locus.

In a study by Zhao and co-workers, they found that by using asymmetric PCR, (where concentration of one primer is 10 times more than the other to facilitate preferential amplification of one strand rather than the other), the sense and antisense strands of short tandem repeats (STR) migrated at different rates, thereby accounting for the shadow bands observed on the denaturing PAGE. In a further expansion of this idea, I put forward a proposal explaining the large number of stutter bands in the thin gel (Fig. 23) as opposed to the thick (1.0 mm) gel (Fig. 26).

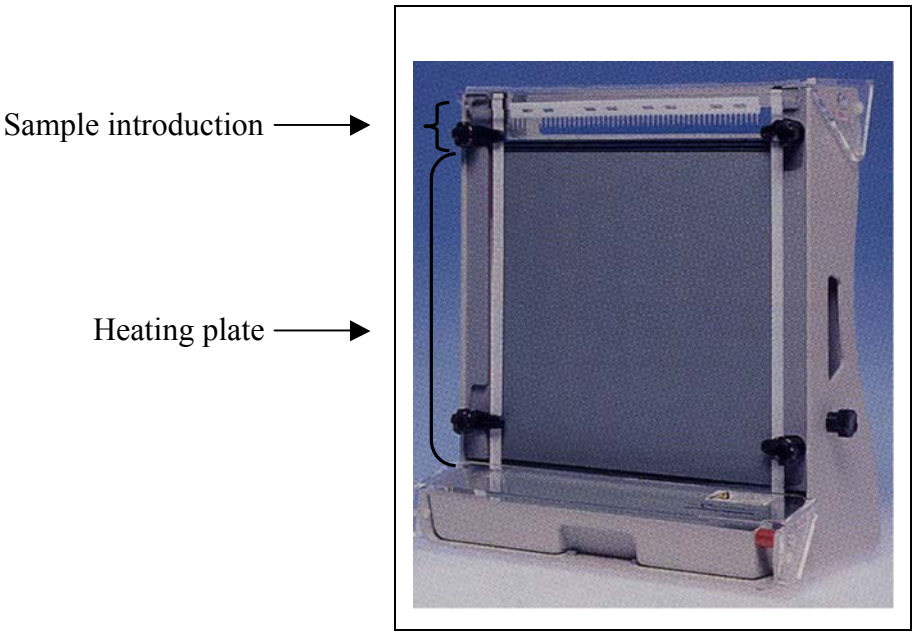

Figure 29. Sequencing gel chamber from GIBCO BRL used for electrophoresis of both thin (0.4 mm) and thick (1.0 mm) gels. Picture taken from the website of GIBCO BRL.

Figure 29 showed the model of the sequencing chamber used for the electrophoresis of both gels. The top section indicated by “sample introduction” is the area where samples are loaded for the electrophoresis. This part of the gel as

compared to the rest of the gel plate, is not in direct contact to the heating plate. This heating plate ensures the uniform distribution of heat during the gel electrophoresis. Therefore, at any time point, the gel region at the sample loading area is possibly cooler than the rest of the gel. Furthermore, when loading many samples, not only does the gel have the opportunity to cool down, this also implies that loaded samples of supposedly separated strands can also re-anneal to each other.

Since a thin gel will heat up faster when the electrophoresis begins, these 're-annealed' strands can start to denature upon entering the gel region where the heating plate is located. However, in a microsatellite PCR reaction, there are not only the sense strands containing e.g., --------CACACACACACA--------, and the antisense strands i.e., --------GTGTGTGTGTGT--------, there are also composites of these strands where mutations during PCR in the repeat or in the flanking regions may have occurred. All these similar strands, upon separating from its complementary strand, may form different secondary structures that migrate at different rates through the gel, thereby accounting for the different number of shadow bands observed (Fig. 25).

Currently only the methods using rapidly amplified polymorphic DNA (RAPD) (Levitan and Grosberg 1993) and amplified fragment length polymorphism (AFLP) (Cadavid et al., 2004) have been successfully used in *Hydractinia*. *Hydractinia* may possess microsatellites but the efforts to find a locus polymorphic enough to allow discrimination between the two chosen, closely related kin failed.

References

Ahuja SK, Lee JC, Murphy PM. 1996. CXC chemokines bind to unique sets of selectivity determinants that can function independently and are broadly distributed on multiple domains of human interleukin-8 receptor B. Determinants of high affinity binding and receptor activation are distinct. Journal Biological Chemistry 271(1):225-232.

Austin TW, Solar SP, Ziegler FC, Liem L, Matthews W. 1997. A role for the Wnt gene family in hematopoiesis: expansion of multilineage progenitor cells. Blood 89(10):3624-3635.

Avise JC. Molecular markers, natural history and evolution. Chapman and Hall. 511pp.

Bain J, McLauchlan H, Elliot M, Cohen P. 2003. The specificities of protein kinase inhibitors: an update. Biochemical Journal 371:199-204.

Bode HR. 1996. The interstitial cell lineage of *hydra*: a stem cell system that arose early in evolution. Journal Cell Science 109:1155-1164.

Brohede J, Ellegren H. 1999. Microsatellite evolution: polarity of substitutions within repeats and neutrality of flanking sequences. Proceedings Royal Society London B 266:825-833.

Buss LW. 1987. Somatic cell parasitism and the evolution of somatic tissue compatibility. Proceedings National Academy Science (17):5337-5341.

Buss LW. 1999. Slime molds, ascidians, and the utility of evolutionary theory. Proccedings National Academy Science 6(16):8801-8803.

Cabrera CV, Alonso MC, Johnston P, Phillips RG, Lawrence PA. 1987. Phenocopies induced with antisense RNA identify the wingless gene. Cell 50:659-663.

Cadavid LF, Powell AE, Nicotra ML, Moreno M, Buss LW. 2004. An invertebrate histocompatibility complex. Genetics 167(1):357-365.

Carron C, Pasca A, Djiane A, Boucaut JC, Shi DL, Umbhauer M. 2003. Frizzled receptor dimerization is sufficient to activate the Wnt/β-catenin pathway. Journal Cell Science 116:2541-2550

Castelo-Branco G, Rawal N, Arenas E. 2004. GSK-3beta inhibition/beta-catenin stabilization in ventral midbrain precursors increases differentiation into dopamine. neurons. Journal of Cell Science 117(Pt 24):5731-5737.

Chakraborty R, Kimmel M, Stivers DN, Davison LJ, Deka R. 1997. Relative mutation rates at di-, tri-, and tetranucleotide microsatellite loci. Proceedings National Academy Science 94(3):1041-1046.

Chen RH, Ding WV, McCormick F. 2000. Wnt signalling to beta-catenin involves two interactive components, Glycogen synthase kinase-3 beta inhibition and activation of protein kinase C. Journal Biological Chemistry 275:17894-17899.

Chenn A, Walsh CA. 2002. Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. Science 297(5580):365-369.

Chenn A, Walsh CA. 2003. Increased neuronal prduction, enlarged forebrains and cytoarchitectural distortions in ß-catenin overexpressing transgenic mice. Cerebral Cortex. Changing concepts of Cortical Development 13(6):599-606.

Christiansen JH, Monkley SJ, Wainwright BJ. 1996. Murine WNT11 is a secreted glycoprotein that morphologically transforms mammary epithelial cells. Oncogene 12:2705-2711.

Clarke LA, Rebelo CS, Goncalves J, Boavida MG, Jordan P. 2001. PCR amplification introduces errors into mononucleotide and dineucleotide repeat sequences. Journal clinical pathology 54:351-353.

Clisson I, Lathuilliere M, Ronau-Roy B. 2000. Conservation and evolution of microsatellite loci in primate taxa. American Journal Primatology 50:205-214.

Crabtree GR, Olson EN. 2002. NFAT signaling: choreographing the social lives of cells. Cell S67-79.

Cook D, Fry MJ, Hughes K, Sumathipala R, Woodgett JR, Dale TC. 1996. *Wingless* inactivates glycogen synthase kinase-3 via an intracellular pathway which involves protein kinase C. EMBO J 15:4526-4536.

Davies LE. 1970. Cell division during defifferntiation and redifferentistion in regenerating isolated gastrodermis of *Hydra*. Experimental Cellular Research 60:127-132.

Diehl JA, Cheng M, Roussel MF, Sherr CJ. 1998. Glycogen synthase kinase-3ß regulates cyclin D1 proteolysis and subcellular localisation. Genes and Development 12:3499-3511.

Dominguez-Caceres MA, Garcia-Martinez JM, Calcabrini A, Gonzalez L, Porque PG, Leon J, Martin-Perez J. 2004. Prolactin induces c-Myc expression and cell survival through activation of Src/Akt pathway in lymphoid cells. Oncogene 23(44):7378-7390.

Du SJ, Purcell SM, Christian JL, McGrew LL, Moon RT. 1995. Identification of distinct classes and functional domains of Wnts through expression of wild-type and chimeric proteins in *Xenopus* embryos. Molecular Cell Biology 15:2625-2634.

Duman-Scheel M, Weng L, Xin S, Du W. 2002. Hedgehog regulates cell growth and proliferation by inducing Cyclin D and Cylin E. Nature 2002. 417:299-304.

Eiben R. Storage of embryonically transcribed poly(A) RNA and its utilization during metamorphosis of the hydroid *Hydractinia echinata*. 1982. Wilhelm Roux's Archives. 191:270-276.

Ellegren H. 2004. Microsatellites: Simple sequences with complex evolution. Nature Reviews Genetics 5: 435-445.

Estoup A, Cornuet JM. 1999. Microsatellite evolution: inferences from population data. In: Goldstein DB, Schlötterer C. (eds). Microsatellites: evolution and applications. Oxford University Press. 352 pp.

Etheridge SL, Spencer GJ, Heath DJ, Genever PG. 2004. Expression Profiling and Functional Analysis of Wnt Signalling Mechanisms in Mesenchymal Stem Cells. Stem Cells 22:849-860.

Extavour CG, Akam M. 2003. Mechanism of germ cell specification across the metazoans: epigenesis and preformation. Development 130:5969-5884.

Frank U, Leitz T, Müller WA. 2001. The hydroid *Hydractinia*: a versatile, informative cnidaria representative. Bioessays 23:963-971.

Fuchs MA, Mokady O, Frank U. 2002. The ontogeny of allorecognition in a colonial hydroid and the fate of early established chimeras. International Journal Developmental Biology 46(5):699-704.

Gautschi B, Muller JP, Schmid B, Shykoff JA. 2003. Effective number of breeders and maintenance of genetic diversity in the captive bearded vulture population. Heredity 91(1):9-16.

Ginsburg M, Snow MHL, McLaren A. 1990. Primordial germ cells in the mouse embryo during gastrulation. Development 110:521-528.

Goldstein DB, Clark AG. 1995. Microsatellite variation in North American populations of *Drosophila melanogaster*. Nucleic Acids Research 23(19):3882-3886.

Goldstein DB, Schlötterer C. 1999. Micorsatellites: evolution and applications. Oxford University Press. 352 pp.

Haynes J, Burnett AL. 1963. Dedifferntiation and redifferentiation of cells in *Hydra viridis*. Science 142:1481-1483.

Hassel M, Bridge DM, Stover NA, Kleinholtz H, Steele RE. 1998. The level of expression of a protein kinase C gene may be an important component of the patterning process in Hydra. Development Genes Evolution 207:502-514.

Hauenschild C. 1954. Genetische und entwicklungsphysiologische Untersuchungen über intersexualität und Gewebeverträglichkeit bei *Hydractinia echinata*. Whilhelm Roux' Archiv für Entwicklungsmechanik Org. 147:1-41.

Hauenschild C. 1956. Über die Vererbung einer Gewebeverträglichkeitseigenschaft bei dem Hydroid polypen *Hydractinia echinata*. Z. Naturforsch. 11:132-138.

He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler.1998. Identification of c-MYC as a target of the APC pathway. Science 281:1509-1512.

Heisenberg CP, Tada M, Rauch GJ, Saude L, Concha ML, Geisler R, Stemple DL, Smith JC, Wilson SW. 2000. Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. Nature 405:76-81.

Hobmayer E, Hatta M, Fischer R, Fujisawa T, Holstein TW, Sugiyama T. 1996. Identification of a *Hydra* homologue of the beta-catenin/plakoglobin/armadillo gene family. Genes 12:155-159.

Hobmayer B, Rentzsch F, Kuhn K, Happel CM, Laue CCV, Snyder P, Rothbächer U, Holstein TW. 2000. WNT signalling molecules act in axis formation in the diploblastic metaozoan *Hydra*. Nature 407:186-189.

Hoffman U, Kroiher M. 2001. A possible role for the cnidarian homologue of serum response factor in decision making by undifferentiated cells. Developmental Biology 236:304-315.

Howe LR, Brown AM. 2004. Wnt signalling and breast cancer. Cancer-biology-and-therapy 3(1): 36-41

Huang HC, Klein PS. 2004. The Frizzled family: receptors for multiple signal transduction pathways. Genome Biology 5:234.

Huelsken J, Behrens J. 2002. The Wnt signalling pathway. Journal of Cell Science 115:3977-3978.

Hsieh JC. 2004. Specificity of WNT-receptor interactions. Frontiers in Bioscience 1(9):1333-1338.

Ikeda S, Kishida S, Yamamoto H, Murai H, Koyama S, Kikuchi A. 1998. Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta-dependent phosphorylation of beta-catenin. EMBO Journal 17:1371-84.

Jia J, Amanai K, Wang G, Tang J, Wang B, Jiang J. 2002. Shaggy/GSK3 antagonizes Hedgehog signalling by regulating Cubitus interruptus. Nature 416:548-552.

Jones N, Ougham H, Thomas H. 1997. Markers and mapping: we are all genetiiciists now. New Phytology 137:165-177.

Knockaert M, Wieking K, Schmitt S, Leost M, Grant KM., Mottram JC, Kunick C, Meijer L. 2002. Intracellular targets of paulllones. Journal Biological Chemistry 277:25493-25501.

Kortschak RD, Samuel G, Saint R, Miller DJ. 2003. EST analysis of the cnidarian *Acropora millepora* reveals extensive gene loss and rapid sequence divergence in the model invertebrates. Current Biology 13(24):2190-2195.

Koyanagi M, Ono K, Suga H, Iwabe N, Miyata T. 1998. Phospholipase C cDNAs from sponge and hydra: antiquity of genes involved in the inositol phospholipids signalling pathway. FEBS Letters 439:66-70.

Kuhl M, Sheldale LC, Malbon CC, Moon RT. 2000. Ca^{2+}/calmodulin-dependent protein kinase II is stimulated by Wnt and Frizzled homologs and promote ventral cell fates.in *Xenopus*. Journal Biological Chemistry 275:12701-12711.

Kunick C, Laurenrot K, Leost M, Meijer L, Lemcke T. 2004. 1-Azakenpaullone is a selective inhibitor of the glycogen synthase kinase-3 beta. Bioorganic Medicinal Chemistry Letters 14:413-416.

Kusserow A, Pang K, Sturm C, Hrouda M, Lentfer J, Schmidt HA, Technau U, von Haeseler A, Hobmayer B, Martindale MQ, Holstein TW. Unexpected complexity of the Wnt gene family in a sea anemone. Nature 433(7022):156-160.

Lahusen T, de Siervi A, Kunick C, Senderowicz AM. 2003. Alsterpaullone, a novel cyclin-dependent kinase inhibitor, induces apoposis by activation of caspase-99 due to perturbation in mitochondrial membrane potential. Molecular Carcinogenesis 36:183-194.

Lai Y, Sun F. 2003. The relationship between microsatellite slippage mutation rate and the number of repeat units. Molecular, Biology and Evolution 20:2123-2131.

Lee HY, Kleber M, Hari L, Brault V, Suter U, Taketo MM, Kemler R, Sommer L. 2004. Instructive role of Wnt/beta-catenin in sensory fate specification in neural crest stem cells. Science 03(5660):1020-1023.

Leeflang EP, Tavare S, Marjoram P, Neal CO, Srinidhi J, MacFarlane H, MacDonald ME, Gusella JF, de Young M, Wexler NS, Arnheim N. 1999. Analysis of germline mutation spectra at the Huntington's disease locus supports a mitotic mutation mechanism. Human Molecular Genetics 8:173-183.

Leost M, Schultz C, Link A, Wu YZ, Biernat J, Mandelkow EM, Bibb, JA, Snyder GL, Greengard P, Zaharevitz DW, Gussio R, Senderowics AM, Sausville EA, Kunick C, Meijer L. 2000. Paullones are potent inhibitors of glycogen synthase kinase-3ß and cyclin-dependent kinase5/p25. European Journal Biochemistry 267:5983-5994.

Levitan DR, Grosberg RK. 1993. The analysis or paternity and maternity in the marine hydrozoan *Hydractinia symbiolongicarpus* using randomly amplified polymorphic DNA (RAPD) markers. Molecular Ecology 2:315-326.

Liepelt S, Kuhlenkamp V, Anzidei M, Vendramin GG, Ziegenhagen B. 2001. Pitfalls in determining size homoplasy of microsatellite loci. Molecular Ecology Notes 1:332-335.

Lindgens D, Holstein TW, Technau U. 2004. *Hyzic*, the *Hydra* homolog of the zic/odd-paired gene, is involved in the early specification of the sensory nematocytes. Development 131:191-201.

Lustig B, Behrens J. 2003. The Wnt signalling pathway and its role in tumor development. Journal of Cancer Research and Clinical Oncology 129:199-221.

Magor BG, De Tomaso A, Rinkevich B, Weissman IL. 1999. Allorecognition in colonial tunicates: protection against predatory cell lineages? Immunological Review 167:69-67.

Malliri A, Collard JG. 2003. Role of Rho-family proteins in cell adhesion and cancer. Current-opinion-in-cell-biology 15(5): 583-9.

Martin VJ, Archer WE. 1997. Stages of larval development and stem cell population changes during metamorphosis of a hydrozoan planula. Biological Bulletin 192:41-52.

Martin VJ, Thomas MB. 1981. Elimination of the interstitial cells in the planula larva of the marine hydrozoan *Pennaria tiarella*. Journal of Experimental Zoology 217:303-323.

McLaren A. 2000. Establishment of germ cell lineage in mammals. Journal of Cellular Physiology 182:141-143.

McMahon AP, Moon RT. 1989. Ectopic expression of the proto-oncogene int-1 in *Xenopus* embryos leads to duplication of the embryonic axis. Cell 58:1075-1084.

Meijer L, Flajolet M, Greengard P. 2004. Pharmacological inhibitors of glycogen synthase kinase 3. Trends Pharmacological Science 25(9):471-80.

Miller JR 2001. The Wnts. Genome Biology 3 Reviews 3001.1-3001.15.

Minobe S, Fei K, Yan L, Sarra MP Jr, Werle MJ. 2000. Identification and characterization of the epithelial polarity receptor "Frizzled" in *Hydra vulgaris*. Development Genes Evolution 210:258-262.

Mochizuk K, Sano H, Kobayashi S, Nishimiya-Fujisawa C, Fujisawa T. 2000. Expression and evolutionary conservation of nanos-related genes in *Hydra*. Development Genes and Evolution 210:591-602.

Mochizuk K, Nishimiya-Fujisawa C, Fujisawa T. 2001. Universal occurrence of the vasa-related genes among metazoans and their germline expression in *Hydra*. Development Genes and Evolution 211:299-308.

Mokady O, Buss LW. 1996. Transmission genetics of allorecognition in *Hydractinia symbiolongicarpus* (Cnidaria:Hydrozoa). Genetics 143(2):823-827.

Morrison SJ, Shah NM, Anderson DJ. 1997. Regulatory mechanisms in stem cell biology. Cell 88:287-298.

Müller WA. 1964. Experimentelle Untersuchungen über Stockentwicklung, polypendifferenzierung und Sexualchimären bei *Hydractinia echinata*. Roux' Archiv für Entwicklungsmechanik 155:181-268.

Müller WA. 1967. Differenzierungspotenzen und Geschlechtsstabilität der I-Zellen von Hydractnia echinata. Roux' Archiv für Entwicklungsmechanik 159:412-432.

Müller WA. 1989. Diacylglycerol-induced multiheaded formation in *Hydra*. Development 105:390-316.

Müller WA. 1996. Developmental biology. Springer-Verlag New York, Inc. 382pp.

Müller WA, Leitz T. 2002. Metamorphosis in the Cnidaria. Canadian Journal of Zoology 80:1755-1771.

Müller WA, Teo R, Frank U. 2004a. Totipotent migratory stem cells in a hydroid. Developmental Biology 275:215-224.

Müller WA, Teo R, Möhrlen F. 2004b. Patterning a multi-headed mutant in *Hydractinia*: enhancement of head formation and its phenotypic normalizaion. International Journal of Developmental Biology 48:9-15.

Munck A. 1986. Zelldifferenzierung bei *Hydractinia echinata* Entstehung und Rolle der Interstitiellen Zellen während der Embryonalentwicklung; Herstellung eines monoklonalen Antikörpers als I-Zell-Marker. Dissertation der Fakultät für Biologie durchgeführt am Zoologischen Institut der Ludwig-Maximilian-Universität München.

Nusse R, Varmus HE. 1982. Many tumours induced by the mouse mammary tumour virus contain a provirus integrated in the same region of the host genome. Cell 31:99-109.

Pancer Z, Gershon H, Rinkevich B. 1995. Coexistence and possible parasitism of somatic and germ cell lines in chimeras of the colonial urochordate *Botryllus schlosseri*. Biological Bulletin 189:106-112.

Peifer M, Polakis P. 2000. Wnt signalling in oncogenesis and embryogenesis-a look outside the nucleus. Science 287:1606-1609.

Pepin L, Amigues Y, Lepingle A, Berthier JL, Bensaid A, Vaiman D. 1995. Sequence conservation of microsatellites between *Bos taurus* (cattle), *Capra hircus* (goat) and related species. Examples of use in parentage testing and phylogeny analysis. Heredity 74 :53-61.

Peter R, Gschwentner R, Schürmann W, Rieger RM, Ladurner P. 2004. The significance of stem cells I free-living flatworms: one common source for all cells in the adult. Journal of Applied Biomedicine 2:21-35.

Plickert G, Kroiher M, Munck A. 1988. Cell proliferation and early differentiation during embryonic development and metamorphosis of *Hydractinia echinata*. Development 103:795-803.

Povelones M, Nusse R. 2002. Wnt signalling sees spots. Nature Cell Biology 4:E249-E250.

Raff M. 2003. Adult stem cell plasticity: Fact or Artifact? Annual Review Cell Developmental Biology (19):1-22.

Rentzsch F, Hobmayer B, Holstein TW. 2005. Glycogen synthase kinase 3 has a proapoptotic function in *Hydra* gametogenesis. Developmental Biology 278:1-12.

Reya T, Duncan AW, Allies L, Domen J, Scherer DC, Willert K, Hintz L, Nusse R, Weissman IL. 2003. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature 423:409-414.

Rijsewijk F, Schuermann M, Wagenaar E, Parren P, Weigel D and Nusse, R. 1987. The *Drosophila* homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless. Cell 50:649-657.

Rinkevich B. 1998. Immunology of human implantation: from the invertebrates' point of view. Human Reproduction 2:455-459.

Rinkevich B, Weissman IL. 1992. Allogeneic resorption in colonial protochordates: consequences of nonself recognition. Developmental Comparative Immunology 16(4):275-286.

Sacks PG, Davies LE. 1979. Production of nerveless *Hydra attenuata* by hydroxyurea treatments. Journal Cellular Science 37:189-203.

Saneyoshi T, Kume S, Amasaki Y. Mikoshiba K. 2002 The Wnt/calcium pathway activates NF-AT and promotes ventral cell fate in Xenopus embryos. Nature 417:295-299.

Schultz C, Link A, Leost M, Zaharevitz DW, Gussio R, Sausville EA, Meijer L, Kunick C. 1999. Paullones, a series of cyclin-dependent kinase inhibitors: synthesis, evaluation of CDK1/cyclin B inhibition, and in vitro antitumor activity. Journal Medicinal Chemistry 42(15):2909-2919.

Seipel K, Yanze N, Schmid V. 2004a. The germ line and somatic stem cell gene in the jellyfish *Podocoryne carnea*. International Journal Developmental Biology 48:1-7.

Seipel K, Yanze N, Müller P, Streitwolf R, Schmid V. 2004b. Basic leusine zipper transcription factors C/EBP and MafL in the hydrozoan jellyfish *Podocoryne carnea*. Developmental Dynamics 230:392-402.

Sharma RP, Chopra VL. 1976. Effect of the Wingless (wg1) mutation on wing and haltere development in *Drosophila melanogaster*. Developmental Biology 48:461-465.

Shimizu H, Julius MA, Giarre M, Zheng Z, Brown AM, KitajewskinJ. 1997. Transformation by Wnt family proteins correlates with regulation of beta-catenin. Cell Growth Differentiation 12:1349-1358.

Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A. 1999. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proceedings National Academy Science 96:5522-5527.

Slusarski DC, Corces VG, Moon RT. 1997. Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling. Nature 390:410-413.

Sokol S. 2000. A role for Wnts in morpho-genesis and tissue polarity. Nature Cell Biology 2:E1-E2.

Stoner D, Rinkevich B, Weissman IR. Heritable germ and somatic cell lineage competitions in chimeric colonial protochordates. Proceedings National Academy of Science 96:9148-9153.

Strutt D. 2003. Frizzled signalling and cell polarisation in *Drosophila* and vertebrates. Development 130(19):4501-4513.

Sturzeneker R, Bevilacqua RA, Haddad LA, Simpson AJ, Pena SD. 2000. Microsatellite instability in tumors as a model to study the process of microsatellite mutations. Human Molecular Genetics 9(3):347-52.

Sunnucks P. 2000. Efficient genetic markers for population biology. Trends in Ecology and Evolution 15:199-203.

Swain RK, Medina A, Steinbeisser H. 2001. Functional analysis of the *Xenopus* frizzled 7 protein domains using chimeric receptors. International Journal Developmental Biology 45:259-264.

Tusándy GE, Simon I. 2001. The HMMTOP transmembrane topology prediction server. Bioinformatics 17:849-885.

Torras R, Yanze N, Schmid V, Gonzalez-Crespo S. 2004. nanos expression at the embryonic posterior pole and the medusa phase in the hydrozoan *Podocoryne carnea*. Evol Dev. (5):362-371.

Torres MA, Yang-Snyder JA, Purcell, SM, DeMarais AA, McGrew LL, Moon RT. 1996. Activities of the Wnt-1 class of secreted signalling factors are antagonized by the Wnt-5A class and by a dominant negative cadherin in early *Xenopus* development. Journal Cell Biology 133:1123-1137.

Umbhauer M, Djiane A, Goisset C, Penzo-Mendez A, Riou JF, Boucaut JC, Shi DL. The C-terminal cytoplasmic Lys-thr-X-X-X-Trp motif in frizzled receptors mediates Wnt/beta-catenin signalling. EMBO 19(18):4944-4954.

Van de Vyer G. 1964. Étude histologique du développement d'*Hydractinia echinata* (Flem.). Cahiers de Biologie Marine V:295-310.

Wagers AJ, Weismann IR. 2004. Plasticity of adult stem cells. Cell 116:639-648.

Wallingford JB, Vogeli KM, Harland RM. 2001. Regulation of convergent extension in *Xenopus* by Wnt 5a and Frizzled-8 is independent of the canonical Wnt pathway. International Journal of Developmental Biology 45:225-227.

Wan QH, Wu H, Fijihara T, Fang SG. 2004. Which genetic marker for which conservation genetics issue? Electrophoresis 25:2165-2176.

Wang HY. 2004. WNT-frizzled signalling via cyclic GMP. Frontiers in Bioscience 1(9):1043-1047.

Weber J.L. 1990. Informativeness of human $(dC\text{-}dA)_n.(dG\text{-}dT)_n$ polymorphisms. Genomics 7:524-30.

Weissman, IR. 2000. Stem cells: Units of development, units of regeneration, and units of evolution. Cell 100:157-168.

Whitehouse AM, Harley EH. 2001. Post-bottleneck genetic diversity of elephant populations in South Africa, revealed using microsatellite analysis. Molecular Ecology 10(9):2139-2149.

Wikramananyake AH, Hong M, Lee PN, Pang K, Byrum CA, Bince JM, Xu R, Martindale MQ. 2003. An ancient role for the nuclear β-catenin in the evolution of axial polarity and germ layer segregation. Nature 426:446-450.

Willert J, Epping M, Pollack JR, Brown PO, Nusse R. 2002. A transcriptional response to Wnt protein in human embryonic carcinoma cells. BMC Developmental Biology 2(1):8

Willert K, Brown JD, Danenberg E, Duncan AW, Weissman IL, Reya T, Yates JR, Nusse R. 2003. Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature 423:448-452.

Winklbauer R, Medina A, Swain RK, Steinbeisser H. 2001. Frizzled-7 signalling controls tissue separation during *Xenopus* gastrulation. Nature 413:856-860.

Wodarz A, Nusse R. 1998. Mechanism of Wnt signalling in development. Annual Review of Cell and Developmental Biology 14:59-88.

Wong GT, Gavin BJ, McMahon AP. 1994. Differential transformation of mammary epithelial cells by Wnt genes. Molecular Cell Biology 14:6278-6286.

Zane L, Bargelloni L, Patarnello T. 2002. Strategies for microsatellite isolation: a review. Molecular Ecology 11:1-16.

Zahareveitz DW, Gussio R, Leost M, Senderowicz AM, Lahusen T, Kunick C, Meijer L, Sausville EA. 1999. Discovery and initial characterization of the paullones, a novel class of small-molecules inhibitors of cyclin-dependent kinases. Cancer Research 59:2566-2569.

Zhao H, Huang S, Li H, Liang C, Yuan B. 1999. A new explanation for shadow bands on denaturing PAGE for products of STRS - the difference between sense and antisense strands in electrophoresis. Zhonghua Yi Xue Yi Chuan Xue Za Zhi 16(5):328-30.

Appendix 1. Names and accession number of Frizzled protein sequences obtained from the NCBI public database used for the CLUSTALW analysis.

>CELEGANS
Definition: wingless protein receptor Cfz2 - Caenorhabditis elegans
Accession Number: T37325

>CIONA
Definition: frizzled homolog [Ciona intestinalis]
Accession Number: BAA92184

>DANIO2
Definition: frizzled 2 [Danio rerio]
Accession Number: NP_571215)

>DANIO7
Definition: Fzd7a protein [Danio rerio]
Accession Number: AAH68322

>DANIO10
Definition: frizzled 10 [Danio rerio]
Accession Number: NP_571211

>DROSOPHILA2
Definition: wingless receptor precursor dfz2 - fruit fly (Drosophila melanogaster).
Accession Number: S71786

>GALLUS10
Definition: Frizzled-10 [Gallus gallus]
Accession Number: AAF61100

>HOMO10
Definition: Frizzled 10 [Homo sapiens]
Accession Number: AAH74998

>HYDRA
Definition: frizzled receptor [Hydra vulgaris]
Accession Number: AAF18154

>MUS5
Definition: frizzled 5 [Mus musculus]
Accession Number: NP_073558

>MUS7
Definition: frizzled homolog 7 (Drosophila) [Mus musculus]
Accession Number: AAH49781

>SUBERITES
Definition: frizzled receptor [Suberites domuncula]
Accession Number: CAD97575

>XENOPUS2
Definition: frizzled-2 [Xenopus laevis]
Accession Number: AAF06359

>XENOPUS5
Definition: frizzled-5 [Xenopus laevis]
Accession Number: AAK51688

>XENOPUS7
Definition: frizzled 7 [Xenopus laevis]
Accession Number: AAD52671

Appendix 2. Results of the CLUSTALW alignment obtained for the sequences above and that of the putative Frizzled sequence from *Hydractinia*.

```
CLUSTAL W (1.82) multiple sequence alignment


Hydra            ------------------------------------------------------------
Hydractinia      ------------------------------------------------------------
XENOPUS2         ------------------------------------------------------------
DANIO2           ------------------------------------------------------------
DROSOPHILA7      RLQWPAGPQGEKAEQHHRGDGRGEQAGTQGAGLAEAWLGLGDVRIGVRPGLEKLGQTLKG 60
XENOPUS7         ------------------------------------------------------------
DANIO7           ------------------------------------------------------------
GALLUS10         ------------------------------------------------------------
HOMO10           ------------------------------------------------------------
DANIO10          ------------------------------------------------------------
MUS5             ------------------------------------------------------------
XENOPUS5         ------------------------------------------------------------
DROSOPHILA2      ------------------------------------------------------------
CELEGANS         ------------------------------------------------------------
CIONA            ------------------------------------------------------------
SUBERITES        ------------------------------------------------------------


Hydra            ------------------------------------------------------------
Hydractinia      ------------------------------------------------------------
XENOPUS2         ------------------------------------------------------------
DANIO2           ------------------------------------------------------------
DROSOPHILA7      NRRSLLLVAAEKAAPLRLGGEGKATPLPSIPHSRPRSLNLRRLRECHAEAAAAPPRLAAS 120
XENOPUS7         ------------------------------------------------------------
DANIO7           ------------------------------------------------------------
GALLUS10         ------------------------------------------------------------
HOMO10           ------------------------------------------------------------
DANIO10          ------------------------------------------------------------
MUS5             ------------------------------------------------------------
XENOPUS5         ------------------------------------------------------------
DROSOPHILA2      ------------------------------------------------------------
CELEGANS         ------------------------------------------------------------
CIONA            ------------------------------------------------------MLESRLQD 8
SUBERITES        ------------------------------------------------------------


Hydra            ------------------------------------------------------------
Hydractinia      ------------------------------------------------------------
XENOPUS2         ------------------------------------------------------------
DANIO2           ------------------------------------------------------------
DROSOPHILA7      PPLCRHPSTLGSAPPTARPRRREDSHAPGAAAPPCIQASPQRLVFFLQLRTPLHSRPAMR 180
XENOPUS7         ------------------------------------------------------------
DANIO7           ------------------------------------------------------------
```

```
GALLUS10        ------------------------------------------------------------
HOMO10          ------------------------------------------------------------
DANIO10         ------------------------------------------------------------
MUS5            ------------------------------------------------------------
XENOPUS5        ------------------------------------------------------------
DROSOPHILA2     ------------------------------------------------------------
CELEGANS        ------------------------------------------------------------
CIONA           TATLCTCKHKEACCERSATLGSRFVSPQFRSVGPSPFTRLNTAVDNIFMQIIKLFRSYLW 68
SUBERITES       ------------------------------------------------------------


Hydra           ---MITITINLMALSLVISCFD----------------YSESAANAECI-------PFAH 34
Hydractinia     --MEIIFTKLFFFVIVLLATVN----------------LSHAGQKTTCV-------SLKH 35
XENOPUS2        ---MQG-VTRASILLIIYHLFT----------------LSLGQLHGEKGI-------SVPE 34
DANIO2          ---MQASGSVCVFLALLLPCCF----------------VASGQHQGDNGI-------AVPD 35
DROSOPHILA7     GPGTAASHSPLGLCALVLALLGALPTD-----------TRAQPYHGEKGI-------SVPD 223
XENOPUS7        ----MSSTVSLLFCGLFLQLC----------------PSAQQYHGEKGI-------SVPD 33
DANIO7          -MQHCCGITRYFLLLFTVALQ----------------TTSGQYHGEKGI-------SIPE 36
GALLUS10        ---MGPAAGNLVRAVLALCWLAEH----------------CAGISSIDI-------ERPG 34
HOMO10          ---MQRPG---PRLWLVLQVMGS----------------CAAISSMDM-------ERPG 30
DANIO10         ---MFAAG---VGLSLGLLCFAGF----------------CSAISSIDP-------DRPG 31
MUS5            MARPDPSAPPSLLLLLLAQLVGR----------------AAAASKAP----------- 31
XENOPUS5        MGSFRSGVFALSFVVLLLDYFAP----------------AQAASKAI----------- 31
DROSOPHILA2     MRHNRLKVLILGLVLLLTSCRADGPLHSADHGMGGMGMGGHGLDASPAPGYGVPAIPKDP 60
CELEGANS        ------MLLRISVLFLLLGSCGA----------------LFGKRQK----------- 24
CIONA           TNKCRGSGVALTFLAMLLASDMLVPTSAVR------YADNGPSLSGRRKPPISGLSGRQG 122
SUBERITES       -----MSLVWFILVLLASGSHSQ----------------QPLCDNQSP----------- 27
                                    .


Hydra           H-------FQICNKMPYNTTMFPNLLEHRN-PEEASLEVAQFKPLVKVGCSDDLEFFLCS 86
Hydractinia     RG------LRLCQNLGYNATMFPNSLEHRN-MENASTELDNFLPLVKIRCAKEIEFFLCS 88
XENOPUS2        HGFCQPISIPLCTDIAYNQTIMPNLLGHTN-QEDAGLEVHQFYPLVKVQCSSELRFFLCS 93
DANIO2          HGFCQPITIPLCTDIAYNQTIMPNLVGHYN-QEDAGLEVHQFYPLVKVQCSPELKFFLCS 94
DROSOPHILA7     HGFCQPISIPLCTDIAYNQTILPNLLGHTN-QEDAGLEVHQFYPLVKVQCSPELRFFLCS 282
XENOPUS7        HGFCQPISIPLCTDIAYNQTIMPNLLGHTN-QEDAGLEVHQFYPLVKVQCSPELRFFLCS 92
DANIO7          HGFCQPISIPLCTDIAYNQTIMPNLLGHTN-QEDAGLEVHQFYPLVKVQCSMDLKFFLCS 95
GALLUS10        DGRCQPIEIPMCKDIGYNMTRMPNLMGHEN-QREAAIQLHEFAPLVEYGCHGHLKFFLCS 93
HOMO10          DGKCQPIEIPMCKDIGYNMTRMPNLMGHEN-QREAAIQLHEFAPLVEYGCHGHLRFFLCS 89
DANIO10         EGRCQEIAIPLCKDIGYNLTVMPNLMGHED-QNEAAIKLHEFAPLIEFGCHSHLKFFLCS 90
MUS5            --VCQEITVPMCRGIGYNLTHMPNQFNHDT-QDEAGLEVHQFWPLVEIHCSPDLRFFLCS 88
XENOPUS5        --VCQEITVPMCKGIGYNHTYMPNQFNHDT-QDEAGMEVHQFWPLVVIQCSLDLKFFLCS 88
DROSOPHILA2     NLRCEEITIPMCRGIGYNMTSFPNEMNHET-QDEAGLEVHQFWPLVEIKCSPDLKFFLCS 119
CELEGANS        ---CEQITIPLCKGIGYNMTSFPNSYGHEK-QEEAGLEVHQFYPLVEVGCFQHLKFFLCT 80
CIONA           TGVCEPIQVPMCIDIGYNFTDMSVSPSYIYDQKEAAQSVIQFGPLTKTKCAEEMKLLVCS 182
SUBERITES       TAFGSCVPAPTACDMLYSSTAYPNNVTTTS-MMSVSRLIAYQPFVNVIDCSPYSQLFICL 86
                          .  : *. *  .          ...  :     :    *    .:::*


Hydra           VYAPVC-LNHGP-LPPCKPLCERARSGCISIMQKFGFIWPENIR-CERYP-DST---PNA 139
Hydractinia     VYLPVC-LDSGP-LPPCREVCERAQRGCIKLMTQYGFPWPEYLR-CSRFP-KKV---EDR 141
XENOPUS2        MYAPVCTVLEQA-IPPCRSICERARHGCEALMNKFGFQWPERLR-CENFP-RHG---AEQ 147
DANIO2          MYAPVCTVLEKA-IPPCRSICERAKQGCEVLMNKFGFQWPEALR-CEHFP-VLG---DGH 148
DROSOPHILA7     MYAPVCTVLDQA-IPPCRSLCERARQGCEALMNKFGFQWPERLR-CENFP-VHG---AGE 336
XENOPUS7        MYAPVCTVLEQA-IPPCRSLCERARQGCEALMNKFGFQWPERLR-CENFP-VHG---AGE 146
```

```
DANIO7          MYAPVCTVLEQA-IPPCRSLCERARQGCEALMNKFGFQWPERLR-CENFP-VHG---AGE 149
GALLUS10        LYAPMCTEQVSTPIPACRVMCEQARLKCSPIMEQFNFKWPDSLD-CSKLPNKND---PNY 149
HOMO10          LYAPMCTEQVSTPIPACRVMCEQARLKCSPIMEQFNFKWPDSLD-CRKLPNKND---PNY 145
DANIO10         LYAPMCTEQVSTPIPACRVMCEQARQKCSPIMEQFNFHWPESLD-CSRLPNKND---PNY 146
MUS5            MYTPICLPDYHKPLPPCRSVCERAKAGCSPLMRQS-FAWPERMS-CDRLPVLGG--DAEV 144
XENOPUS5        MYTPICLPDYRKPLPPCRSVCERAKAGCSPLMRKYGFAWPERMN-CDRLPEHG---DPDT 144
DROSOPHILA2     MYTPICLEDYHKPLPVCRSVCERARSGCAPIMQQYSFEWPERMA-CEHLPLHG---DPDN 175
CELEGANS        MYTPICQENYDKPILPCMELCVEARSKCSPIMAKYGFRWPETLS-CEALPKMSDQMSTGN 139
CIONA           VYTPICIPGYPGFLPPCRFICEAAKAGCEPILKKYDRTWPNLFD-CKQFPDSQG---KNP 238
SUBERITES       SLAPVCTSGSPR--HPCESLCDLVLASCSDKIQQLGLPDTDCLLNCDRYPSTGCVDTDDP 144
                   *:*          *  :*  .   *   : :     .: :  *   *

Hydra           LCIDN------AETKKP-----------------------------EEKVRVK------ 157
Hydractinia     LCVDKPFKEPGTNGNNG-----------------------------NNGINTGGIPVNP 171
XENOPUS2        ICVGQNHSEDGG---------------------------------PTLLTTSPPHHGT 172
DANIO2          ICVGQNDS---M---------------------------------ATVSPVHMPIPGT 170
DROSOPHILA7     ICVGQNTSDGSGGAGGS----------------------------PTAYPTAPYLPDP 366
XENOPUS7        ICVGQNTSDNSPSG-------------------------------PTARP-SPYLPDS 172
DANIO7          ICVGQNTSDAGS---------------------------------PTSNP-TPYVPEL 173
GALLUS10        LCMEAPNNG------------------------------------SDEPPRGSSMLPP 171
HOMO10          LCMEAPNNG------------------------------------SDEPTRGSGLFPP 167
DANIO10         LCMEAPNNG------------------------------------TDEPPKGSHTQSP 168
MUS5            LCMDYN-RSEATTASPK----------------------------SFPAKPTLPG--- 170
XENOPUS5        LCMYYN-WTETTTTLPP----------------------------THPPKVKTP---- 169
DROSOPHILA2     LCMEQPSYTEAGSGGSSGGSGGSGSGSGSGGKRKQGGSGSGGSGAGGSSGSTSTKPCRGR 235
CELEGANS        ICAAPPDTPKKQHKGHHHKNQNQ----------------------NQNQNHNYSPDGP 175
CIONA           PCLHFNRSATEEPAVPR----------------------------TITKKGESKTTPGG 269
SUBERITES       LVLAIQSIN------------------------------------------------- 153


Hydra           --NKTQSSYND------YRCKTDQQK-DAKHYN-------------------------FM 183
Hydractinia     SHNTTHATYDD------YRCPAKQQK-ETKHYK-------------------------FM 199
XENOPUS2        PGPPIYATLDH-----PFHCPRVLKVPSYLNYR-------------------------FL 202
DANIO2          PSVQLYSTPDK-----PFRCPSTLKVPAYLSYK-------------------------FL 200
DROSOPHILA7     PFTAMSPSDGRGRLSFPFSCPRQLKVPPYLGYR-------------------------FL 401
XENOPUS7        --ITFQPHPHR-----DFTCPRQLKVPPYLAYR-------------------------FL 200
DANIO7          --ITLQPNLVVRP-NQQFTCPLQLKVPTYLKYH-------------------------FM 205
GALLUS10        MFRPQRPSTGHDLQQHKDSLSRTSCENPGKFHH-------------------------VE 206
HOMO10          LFRPQRPHSAQEHPLKDGGPGRGGCDNPGKFHH-------------------------VE 202
DANIO10         DSRPPRPGNSQELPIKER-VGKTTCSNPGKFHY-------------------------VQ 202
MUS5            -PPGAPSSGGECPSGGPSVCTCREPFVPILKES--------------HPLYN---KVRTG 212
XENOPUS5        ------------TSDCDGVCKCREPFVSITRES--------------HPLYN---RIKTG 200
DROSOPHILA2     NSKNCQNPQGEKASGKECSCSCRSPLIFLGKEQLLQQQSQMPMMHHPHHWYMNLTVQRIA 295
CELEGANS        EVGISKIDNEVIAGPSECQCTCNQPFQFVASEK-----------------------SKVG 212
CIONA           PVASVTHHPYKPINGCPCACARRMVKITNKKDPLYG-------------------KVTTG 310
SUBERITES       ----------------PLTTIPAITQTPHTTEP--------------------------- 170


Hydra           GKPGCASECDP-------VYFTGKEKDFARNWVLFWSVVCLISTAFTLVTFFCLICQGSL 236
Hydractinia     GTEKCASLCTP-------IYFTHKEKDFARNWVLFWSVVCMLSTAFTLLAFIVDMPRFRY 252
XENOPUS2        GEKDCAAPCEPTKSD-GFMFFSQDEIRFARIWILIWSVLCCASTFITVTTYLVDMQRFRY 261
DANIO2          GEPDCGAPCESSRTHRAYMFFTDQEIEFARIWILIWSSLCCASTLFTVTTYIVDMQRFKY 260
DROSOPHILA7     GERDCGAPCEPGRAN-GLMYFKEEERRFARLWVGVWSVLCCASTLFTVLTYLVDMRRFSY 460
```

```
XENOPUS7        GEKDCGAPCEPGKAN-GLMYFKEEEVRFARLWVGIWAILCCISTLFTVLTYLVDMRRFSY 259
DANIO7          GEKDCGAPCEPTRPN-GLMYFREEEVKFGRLWVGIWSILCCVSTLFTVLTYLVDMRRFRY 264
GALLUS10        KSASCAPLCTPG----VDVYWSKDDKQFAVIWIAIWSILCFFSSAFTVLTFLIDPQRFKY 262
HOMO10          KSASCAPLCTPG----VDVYWSREDKRFAVVWLAIWAVLCFFSSAFTVLTFLIDPARFRY 258
DANIO10         KSESCAPKCYSN----VDVYWSQGDKRFSMVWIAIWSILCFISSAFTVLTFLIDPQRFKY 258
MUS5            QVPNCAVPCYQP-------SFSPDERTFATFWIGLWSVLCFISTSTTVATFLIDMERFRY 265
XENOPUS5        QVPNCAMPCFQP-------YFTQDEKMFVTFWIGLWSILCFISTFTTVATFLIDMERFRY 253
DROSOPHILA2     GVPNCGIPCKGP-------FFSNDEKDFAGLWIALWSGLCFCSTLMTLTTFIIDTERFKY 348
CELEGANS        NVTNCAYSCHSP-------ALAES-HSLVSNWMAFWSITCCVLASFTFLTFLIETDRFQY 264
CIONA           GVPNCAMPCKSP-------YFSEEKRKFAESWITVWSVVCFAITMLTVGTYLIDTQRFAY 363
SUBERITES       STANSSGECPEVS------YFTDSAKSFAQGWLAFWALICFISTTLTIATFVLDPSRFQY 224
                    ..  *                :   *: .*:  *   :  *. ::.    :

Hydra           SSKTYYIFIRCYFMISIAFFMWSLSDNAIACHK--TDKGVELLNQGTENAACTVDFMFIY 294
Hydractinia     PERPIIFLSGCYFIVAIAFISGPVSDNAIACHK--TDDGTQLLNQGTDNASCTIVFMLIY 310
XENOPUS2        PERPIIFLSGCYTMVSVAYIAGFVLGDKVVCNEGFSEDGYKTVVQGTKKEGCTILFMMLY 321
DANIO2          PERPIIFLSGCYTMVSIAYIAGYFLGDKVVCNESFFPDSYKTIVQGTKKEGCTILFMMLY 320
DROSOPHILA7     PERPIIFLSGCYFMVAVAHVAGFLLEDRAVCVERFSDDGYRTVAQGTKKEGCTILFMVLY 520
XENOPUS7        PERPIIFLSGCYFMVAVAYTAGFLLEERAVCVERFSEDSYRTVAQGTKKEGCTILFMILY 319
DANIO7          PERPIIFLSGCYFMVAVAYAAGFFLEDKVVCIDKFSDDGYKTVAQGTKKEGCTILFMILY 324
GALLUS10        PERPIIFLSMCYCVYSVGYIIRLFSGAESIACDR-DSGQLYVIQEGLESTGCTIVFLVLY 321
HOMO10          PERPIIFLSMCYCVYSVGYLIRLFAGAESIACDR-DSGQLYVIQEGLESTGCTLVFLVLY 317
DANIO10         PERPIIFLSMSYCVYSVGFLVRLFVGVENVACDR-DTGVQYIIQEGLESTGCTIVFLILY 317
MUS5            PERPIIFLSACYLCVSLGFLVRLVVGHASVACSR---EHSHIHYETTGPALCTVVFLLVY 322
XENOPUS5        PERPIIFLSACYLFVSIGYVVRLIVGHENVACNK---DH--IHYETTGPALCTIVFLLIY 308
DROSOPHILA2     PERPIVFLSACYFMVAVGYLSRNFLQNEEIAC-----DGLLLRESSTGPHSCTLVFLLTY 403
CELEGANS        PERPIFMLAFCQLMVAVGFMIRYFVGHEEIACDS---MRIKGADDNSG-SLCFVVFLLTY 320
CIONA           PERPIIFLSACYMFVSIGFIIRVVAGHEAVACAW---HSDRVLYQTTGPFLCSIVFLLLY 420
SUBERITES       PWRPVVYLSLSFNLHSLTHFFSKGLGRDLVTCPG-NQFVQTSITWTWGHTPCILVFGLLH 283
                . :.   :  .    :: .                                 * : * . :

Hydra           FFLMSASIWWVILTLTWFLAAGLKWGHEAIEASAQYFHAAAWAIPAAKTIAILAMNQVDG 354
Hydractinia     FFLMSASIWWVILTLTWFLAAGLKWGHEAIEGSSQYFHAAAWAIPAAKTIAVLAMNQIDG 370
XENOPUS2        FFSMASSIWWVILSLTWFLAAGMKWGHEAIEANSQYFHLAAWAVPAVKTITILAMGQIDG 381
DANIO2          FFSMASSIWWVILSLTWFLAAGMKWGHEAIEANSQYFHLAAWAVPAVKTISILAMGQIDG 380
DROSOPHILA7     FFGMASSIWWVILSLTWFLAAGMKWGHEAIEANSQYFHLAAWAVPAVKTITILAMGQVDG 580
XENOPUS7        FFGMASSIWWVILSLTWFLSAGMKWGHEAIEANSQYFHLAAWAVPAVKTITILAMGQVDG 379
DANIO7          FFGMASSIWWVILSLTWFLSAGMKWGHEAIEANSQYFHLAAWAVPAVKTITILAMGQVDG 384
GALLUS10        YFGMASSLWWVILTLTWFLAAGKKWGHEAIEANSSYFHLAAWAIPAVKTIMILVMRRVAG 381
HOMO10          YFGMASSLWWVVLTLTWFLAAGKKWGHEAIEANSSYFHLAAWAIPAVKTILILVMRRVAG 377
DANIO10         YFGMASSLWWVILTLTWFLAAGKKWGHEAIEANSSYFHLAAWAIPAIKTIMILVMRKVAG 377
MUS5            FFGMASSIWWVILSLTWFLAAGMKWGNEAIAGYAQYFHLAAWLIPSVKSITALALSSVDG 382
XENOPUS5        FFGMASSIWWVILTFTWFLAAGMKWGNEAIASYSQYFHMAAWLIPSVKSIAVLALSSVDG 368
DROSOPHILA2     FFGMASSIWWVILSFTWFLAAGLKWGNEAITKHSQYFHLAAWLIPTVQSVAVLLLSAVDG 463
CELEGANS        FFGMAASVWWVILSLTWVLSAASKWSPEAISSFSFHFHVVGWCLPAIQTVLVIVFNAIDG 380
CIONA           FFGMAGALWWVILSLTWFLAAGLKWGSEAIASYSQYFHAAAWLIPALKSITVLAMSSVDG 480
SUBERITES       ETMMTAFLWWLMLSFSWFLASAFSWSSEAVSHLAPFYHVIAWVLPLVLTIILVATRVVGA 343
                   *:. :**::*:::*.*::. .*. **:   : .:*  .* :*   ::  :    : .

Hydra           DLLSGICFVG----GKNLSALRGFLLAPLFFYLVAGSVFLLAGFVALIRIRKVLKTDHS- 409
Hydractinia     DILAGVCFVG----GKNVNALRGFVLAPLFVYLVVGSFFLFAGFISLIQIRKVLKDDSS- 425
XENOPUS2        DLLSGVCFVG----LNNIDPLRGFVLAPLFVYLFIGTSFLLAGFVSLFRIRTIMKHDG-- 435
DANIO2          DVLSGVCFVG----LNNSDALRGFILAPLFLYLFIGTSFLLAGFVSLFRIRTIMKHDG-- 434
```

```
DROSOPHILA7     DLLSGVCYVG----LSSVDALRGFVLAPLFVYLFIGTSFLLAGFVSLFRIRTIMKHDG-- 634
XENOPUS7        DVLSGVCYVG----INSVDSLRGFVLAPLFVYLFIGTSFLLAGFVSLFRIRTIMKHDG-- 433
DANIO7          DTLTGVCYVG----IYNVDSLRGFVLAPLFVYLFIGTSFLLAGFVSLFRIRTIMKHDG-- 438
GALLUS10        DELTGLCYVG----SMDVNALTGFVLIPLACYLIIGTSFILSGFVALFHIRRVMKTGG-- 435
HOMO10          DELTGVCYVG----SMDVNALTGFVLIPLACYLVIGTSFILSGFVALFHIRRVMKTGG-- 431
DANIO10         DELTGVCYVG----SMDVKALTGFVLIPLSCYLIIGTSFLLSGFVALFHIRKVMKTEG-- 431
MUS5            DPVAGICYVG----NQNLNSLRGFVLGPLVLYLLVGTLFHLAGFVSLFRIRSVIKQ---- 434
XENOPUS5        DPVAGICYVG----NQNLDNLRGFVLAPLVVYLFSGTMFLLAGFVSLFRIRSVIKQ---- 420
DROSOPHILA2     DPILGICYVG----NLNPDHLKTFVLAPLFVYLVIGTTFLMAGFVSLFRIRSVIKQQGGV 519
CELEGANS        DPITGICYVG----NTDLQFQRIFVLFPLLVYFIVGVLFLVIGFCNLWSIRNEVQKQHP- 435
CIONA           DVLSGICFVG----NQSTKTLRGFVLAPLVVYLALGGLFLFLGFVNLFRIRTSIKKVG-- 534
SUBERITES       DELTATCFIVRDENDMSFYALLLGVIIPLVACLITGVVFLVIGFVSLFRIHSIMRHSG-- 401
                * : . *::       .       :: **   :  *  * . **  *  *:  ::

Hydra           ---LRTDKLTKLMVRIGIFSLLYSLPAIIVIACLFYEQSYRLVWDKSWISAWLLMSESCG 466
Hydractinia     ---LRAEKLTRLMVRIGIFSILYSLPAIIVIACLFYEQTYRIEWDSSWISAWLTMLGKCD 482
XENOPUS2        ---TKTEKLERLMVRIGVFSVLYTVPATIVIACYFYEQAFREHWERSWV---SQNCKSLA 489
DANIO2          ---TKTEKLERLMVRIGVFSVLYTVPATIVIACFFYEQAFRQQWEKSWI---SMNCKSLA 488
DROSOPHILA7     ---TKTEKLEKLMVRIGVFSVLYTVPATIVLACYFYEQAFREHWERTWL---LQTCKSYA 688
XENOPUS7        ---TKTEKLEKLMVRIGVFSVMYTVPATIVLACYFYEQAFRDTWEKTWL---VQTCKGYA 487
DANIO7          ---TKTEKLEKLMVRIGVFSVLYTVPATIVIACYFYEQAFREQWEKTWR---MQTCKRFA 492
GALLUS10        ---ENTDKLEKLMVRIGVFSVLYTVPATCVIACYFYERLNMDYWKIVAS---QQKCKMNN 489
HOMO10          ---ENTDKLEKLMVRIGLFSVLYTVPATCVIACYFYERLNMDYWKILAA---QHKCKMNN 485
DANIO10         ---ENTDKLEKLMVRIGVFSVLYTVPATCVIACYFYERLNMDYWKILAG---EQKCADDG 485
MUS5            -GGTKTDKLGKLMIRIGIFTLLYTVPASIVVACYLYEQHYRESWEAALT----CACPGP- 488
XENOPUS5        -GGTKTDKLEKLMIRIGIFSVLYTVPATIVVACYIYEQHYREHWEKTHN----CSCPG-- 473
DROSOPHILA2     GAGVKADKLEKLMIRIGIFSVLYTVPATIVIGCYLYEAAYFEDWIKALA----CPCAQ-- 573
CELEGANS        -SLESAHKITQLMSKIGIFSLLYTIPSLLIICVLFYEQNHRSLWEQSQL----CSCSP-- 488
CIONA           ---KKTDTLEKLMGRIGLFSLLYMVPSAALVACYFYEQQNRELWAKAYN------CRSFH 585
SUBERITES       -KATESSILEKLMIRIGIFVVVFILPAVIVVGCFMYELISRPFWTPISEP---------- 450
                     :  : :** :**:* ::: :*:  ::   :**      *

Hydra           GQSCYHFVGE-DRPDFAVFMIKYLMLLMVGITSGFWIWSGKTIQSWKQFYYKKILNKRPP 525
Hydractinia     EKVCFEFSGK-SRPDFAVFMIKYLMLLMVGITSGFWIWSGKTIQSWRRFYYNKILHQRVP 541
XENOPUS2        IPCPLQYTPR-MTPDFTVYMIKYLMTLIVGITSGFWIWSGKTLHSWRKFYTRLTNSKH-- 546
DANIO2          IPCPMQPAPH-MTPDFTVFMIKYLMTLIVGITSGFWIWSGKTLHSWQKFYVRLTSAGQ-- 545
DROSOPHILA7     VPCPPGHFSP-MSPDFTVFMIKYLMTMIVGITTGFWIWSGKTLQSWRRFYHRLSHSSK-- 745
XENOPUS7        VPCPNYNFAP-MSPDFTVFMIKYLMTMIVGITSSFWIWSGKTLQSWRRFYHRLSNGSK-- 544
DANIO7          VPCPINNFAP-MSPDFTVFMIKYLMTMIVGITSGFWIWSGKTLQSWRRFYKRLSNSNQ-- 549
GALLUS10        QTKNLDCMMNNSIPAVEIFMVKIFMLLVVGITSGMWIWTSKTLQSWQNVCSRRLKKRSRR 549
HOMO10          QTKTLDCLMAASIPAVEIFMVKIFMLLVVGITSGMWIWTSKTLQSWQQVCSRRLKKKSRR 545
DANIO10         KSG-EECVMKSSIPAVEIFMVKIFMLLVVGITSGMWIWTSKTLQSWQNVFSRKLKKKTRR 544
MUS5            -----DAGQPRAKPEYWVLMLKYFMCLVVGITSGVWFWSGKTLESWRRFTSRCCCSSRRG 543
XENOPUS5        -------DKQRYRPDYAVFMLKYLMCLVVGITSGVWIWSGKTLESWKRFTGRCCRNSK-- 524
DROSOPHILA2     ------VKGPGKKPLYSVLMLKYFMALAVGITSGVWIWSGKTLESWRRFWRRLLGAPDRT 627
CELEGANS        -------KQTIGDSSLIISLIKTCCMCILGWTSGFWVCSTKTLSSWK------------- 528
CIONA           GRD-RCSHGNANGPEFAVFIVKYSMTLLIGITNGVWILSGKTITSWRKFYRRCYGCLCGK 644
SUBERITES       -------CNDCVLGNPAVFMTRLFMFLLTGILTGVWIWSKKTLLSWRNLPQRLTTCCRPK 503
                             : : :       *  ...*. : **: **:

Hydra           ---------------------------------KK-IIFKDVSVLTNLTGKTTI------ 545
Hydractinia     ---------------------------------RQYYESKDGSVFTARTNLT-------- 560
XENOPUS2        ---------------------------------------GETTV---------------- 551
```

```
DANIO2          ---------------------------------------GETTV---------------- 550
DROSOPHILA7     ---------------------------------------GETAV---------------- 750
XENOPUS7        ---------------------------------------GETAV---------------- 549
DANIO7          ---------------------------------------GETTV---------------- 554
GALLUS10        ---------------------------------KPASVITSSGIYKKPQHPQKTHLAKYE 576
HOMO10          ---------------------------------KPASVITSGGIYKKAQHPQKTHHGKYE 572
DANIO10         ---------------------------------KAACVFTGSGPYLKPHPALKGHKTKYE 571
MUS5            --------------------------HKSGGAMAAGDYAEASAALTGRTGPPGPTAAYHK 577
XENOPUS5        -------------------------------PINASAYSEASRALTPRTGLSNLTLP-HK 552
DROSOPHILA2     GANQALIKQRPPIPHPYAGSGMGMPVGSAAGSLLATPYTQAGGASVASTSHHHLHHHVLK 687
CELEGANS        ------------------------------------------NAICCLVFLKEKFLQ 543
CIONA           YGLKAKQEGEPLAADDMSKASDNSSGKRTKSDKASDKTSPEEIPLTNSVATSSFTGNPHS 704
SUBERITES       -----------------------------------PVDVPHAVNVGVGEQITMELESSD 527

Hydra           ------------------------------------------------------------
Hydractinia     ------------------------------------------------------------
XENOPUS2        ------------------------------------------------------------
DANIO2          ------------------------------------------------------------
DROSOPHILA7     ------------------------------------------------------------
XENOPUS7        ------------------------------------------------------------
DANIO7          ------------------------------------------------------------
GALLUS10        STLQPPTCV---------------------------------------------------- 585
HOMO10          IPAQSPTCV---------------------------------------------------- 581
DANIO10         PAGPPATCV---------------------------------------------------- 580
MUS5            QVSLSHV------------------------------------------------------ 584
XENOPUS5        QVPLSHV------------------------------------------------------ 559
DROSOPHILA2     QPAASHV------------------------------------------------------ 694
CELEGANS        IILISQI------------------------------------------------------ 550
CIONA           VGGSESKESRSSSARKKSKTQSNGVQHNRNDMYYECNEVDRNDVNQIRHAACDCDIAPCP 764
SUBERITES       KL------------------------------------------------------------ 529

Hydra           ------------------------------------------------------------
Hydractinia     ------------------------------------------------------------
XENOPUS2        ------------------------------------------------------------
DANIO2          ------------------------------------------------------------
DROSOPHILA7     ------------------------------------------------------------
XENOPUS7        ------------------------------------------------------------
DANIO7          ------------------------------------------------------------
GALLUS10        ------------------------------------------------------------
HOMO10          ------------------------------------------------------------
DANIO10         ------------------------------------------------------------
MUS5            ------------------------------------------------------------
XENOPUS5        ------------------------------------------------------------
CELEGANS        ------------------------------------------------------------
CIONA           LPSEVRGNPAKQLSFPEGFETLPDPLDVSTTELEAANRVQHIHHHHHHHHVTQPPCSCGCH 824
SUBERITES       ------------------------------------------------------------

Hydra           ---------------------------------------------
Hydractinia     ---------------------------------------------
XENOPUS2        ---------------------------------------------
```

```
DANIO2          ---------------------------------------------
DROSOPHILA7     ---------------------------------------------
XENOPUS7        ---------------------------------------------
DANIO7          ---------------------------------------------
GALLUS10        ---------------------------------------------
HOMO10          ---------------------------------------------
DANIO10         ---------------------------------------------
MUS5            ---------------------------------------------
XENOPUS5        ---------------------------------------------
DROSOPHILA2     ---------------------------------------------
CELEGANS        ---------------------------------------------
CIONA           ASRVTTRRPTPRRHSHHDPGACYEANHRKLVLTPPAYRTSPTTHSLRR 872
SUBERITES       ---------------------------------------------
```