Identification and characterization of downstream elements of *Hydra* FGFR signaling

Dissertation

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Declaration

The present work has been performed from October 2011 until September 2014 in the group of Prof. Dr. Monika Hassel, Department of Morphology and Evolution of Invertebrates, Philipps University Marburg.

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Isolation and expression analysis of genes encoding potential docking proteins for *Hydra* FGFR

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Sprouty2, a negative regulator of RTK signaling is coexpressed with both FGFRs at the *Hydra* bud base

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Erklärung

Ich versichere, dass ich meine Dissertation mit dem Titel

“Identification and characterization of downstream elements of *Hydra* FGFR signaling”

unter der Leitung von Frau Prof. Dr. Monika Hassel (Fachbereich Biologie, Philipps- Universität Marburg) selbstständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form bei keiner anderen Hochschule eingereicht und hat keinen sonstigen Prüfungszwecken gedient.

Marburg, _____

Ashwini Suryawanshi

Dedicated to my family…

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Summary

Hydra polyps predominantly reproduce through budding in the lower half of the parent's body column. FGFRa (Kringelchen), a member of FGF receptor tyrosine kinases, plays an essential role and controls bud detachment from the parent. Whether signal transduction through *Hydra* FGFR is comparable to FGFR signaling in vertebrate and fly is unknown. In both Bilateria, activated FGFRs recruit docking proteins to connect to downstream pathways and negative regulators. While vertebrates use FRS2 to dock FGFR to the Ras/MAPK or PI3K pathways, a completely unrelated protein, Downstream-of-FGFR (Dof/Stumps/Heartbroken), fulfills this function in *Drosophila*. In *Drosophila,* Dof couples FGFR to MAPK signaling and transcriptionally activates the negative regulator Sprouty (Spry). Spry proteins are necessary to modulate receptor tyrosine kinase activity by interfering with MAPK signaling downstream of RTK.

To elucidate potential downstream signaling elements of ancestral FGFRs, I analyzed genomic and EST sequence databases and identified Spry, FRS2, and/or Dof proteins in phyla derived early from the main lineage of animals – including *Hydra*. Dof was found only within the Eumetazoa, while FRS2 proteins were also predicted in Metazoa and their sister taxon, the Choanoflagellata. For the known FRS2 proteins of Deuterostomia and Ecdysozoa an N-terminal myristoylation site, a PTB domain and multiple C-terminal Grb2 and Shp2 binding sites are typical. This structure also applies to FRS2 in Choanoflagellata and sponges (Porifera). A deviating domain structure of FRS2 proteins is predicted in Placozoa, Cnidaria, and Spiralia (Annelida, Mollusca). Their FRS2 proteins all carry an N-terminal PH, a PTB domain and few Grb2 and Shp2 binding sites. Phylogenetic analysis identified a novel protein family (PH-FRS2). Expression analysis of Dof and FRS2 in *Hydra* revealed high levels of Dof transcripts in the upper body region and the tentacle zone. FRS2 mRNA, in contrast, was detected only weakly at the tentacle bases. The presence of both putative docking proteins in Metazoa hints to an early toolkit for the transduction of FGFR signals. Their functional significance remains to be shown.

I also identified four *spry* genes in *Hydra*, all positioned in the basal most position of the phylogenetic tree. They encode the typical features of Spry proteins namely a c-Cbl TKB (Tyrosine kinase binding) site, a Raf1-binding and a Spry domain. Transcripts of *spry2* were detected at the bud base adjacent to *Hydra FGFRa* from mid to late stages. Since no spry-encoding genes were found in the genomes of Parazoa (*Trichoplax*), sponges (*Oscarella, Amphimedon*), or choanoflagellates (*Salpingoeca*), Sprouties might have occurred in the Cnidaria first - or been lost from the early derived taxa.

Tissue dynamics and the spatiotemporal expression pattern of *FGFRa, dof and spry2*, reveals (a) *spry2* and *FGFRa* are present in the same cells at the bud base, (b) co-expression of *FGFRa* (weakly) and *dof* (strongly) in the head region.

In summary, data suggest the existence of two FGFR pathways. The first pathway functions in bud detachment, as shown previously, and potentially links FGFRa to a negative feedback loop activating Spry2. A second pathway function for FGFR signaling in *Hydra* might be at the bud and at the adult head – in a zone, where the mRNA encoding the docking protein Dof is expressed at a high level, and where the two FGFRs are transcribed at a low level. Here, FGFR might function to control or modulate cell migration towards the head and/or cell differentiation necessary to form and maintain a fully functional head. Further elucidation of these potential functions and the molecular network, in which *Hydra* FGFRs act, is a very interesting task for the future.

Zusammenfassung

Die Fortpflanzung des Süßwasserpolypen *Hydra* geschieht hauptsächlich durch Knospung in der unteren Körperhälfte. Der FGF Rezeptor (FGFR) Kringelchen eine Rezeptortyrosinkinase (RTK) spielt eine wichtige Rolle während der Knospung und kontrolliert die Ablösung der Knospe. Unklar ist, ob die FGFR Signalweiterleitung in *Hydra* ähnlich wie bei Vertebraten und Insekten abläuft. Aktivierte FGFRs der Bilateria rekrutieren Docking Proteine, was zu einer Weiterleitung des Signals an nachgeschaltete Signalkaskaden und negative Regulatoren führt. Vertebraten nutzen hierbei FRS2 zur Weiterleitung des Signals an RAS/MAPK oder PI3K Signalkaskaden, während das nicht-verwandte Protein Downstream-of-FGFR (Dof/Stumps/Heartboken) diese Aufgabe in *Drosophila* übernimmt. Bei *Drosophila* koppelt Dof FGFR an den MAPK Signalweg und aktiviert somit transkriptionell den negativen Regulator Sprouty. Sprouty Proteine interagieren mit dem RTK-nachgeschalteten MAPK Signalweg und sind dadurch in der Lage, das Signal abzuschwächen.

Um mehr über die potentiellen Signalelemente anzestraler FGFRs zu erfahren habe ich Datenbanken von genomischen und EST-Sequenzen untersucht. Hierbei konnte ich Sprouty, FRS2 und Dof Proteine codierende Sequenzen in früh abgeleiteten Tierstämmen, inklusive *Hydra*, identifizieren. Dof war nur in den Eumetazoa vorhanden, während FRS2 ebenfalls in Metazoa und deren Schwestertaxa, den Choanoflagellaten, auffindbar war. In Deuterostomia und Ecdysozoa sind eine N-terminale Myristoylierungsstelle, eine PTB Domäne sowie multiple C-terminale GRB2 und SHP2 Bindestellen typisch, gleiches gilt für FRS2 in Choanoflagellaten und Schwämmen (Porifera). Abweichende Domänenstrukturen für FRS2 Proteine werden für Placozoa, Cnidaria und Spiralia (Annelida, Mollusca) vorhergesagt, deren FRS2 Proteine alle eine N-terminales PH Domäne, eine PTB Domäne und wenige potentielle Grb2 und Shp2 Bindestellen beinhalten. Durch phylogenetische Analysen konnte eine neue Proteinfamilie (PH-FRS2) identifiziert werden. Expressionsanalysen von Dof und FRS2 in *Hydra* zeigten ein hohes Dof-Transkriptionslevel in der oberen Körperregion und der Tentakelzone. Im Gegensatz dazu wurde FRS2 mRNA nur extrem schwach an der Tentakelbasis nachgewissen. Das Vorhandensein beider vermuteter Dockingproteine bei

Metazoa lässt darauf schließen, dass diese zu einem früh entstandener Werkzeugkasten (*toolkit*) für die Weiterleitung von FGFR Signalen gehören. Die funktionelle Bedeutung muss zukünftig gezeigt werden.

Ich konnte vier Spry Gene in *Hydra* identifizieren, die alle phylogenetisch basal eingeordnet werden können. Sie codieren für die typischen Sprouty-Proteineigenschaften, nämlich eine c-Cbl, TKB (*Tyrosine kinase binding*) Domäne, eine Raf1-Bindestelle und eine Sprouty-Domäne. Die Transkription von Spry2 konnte an den Knospenbasen angrenzend zu der Transkription von *Hydra* Kringelchen in mittleren bis späten Knospenstadien detektiert werden. Das Fehlen von Sprouty-codierenden Genen in den Genomen von Parazoa (*Trichoplax*), Schwämmen (*Oscarella, Amphimedon*) oder Choanoflagellaten (*Salpingoeca*) weist darauf hin, dass Sproutys erstmals in Cnidaria auftauchten.

Gewebedynamiken sowie die räumlich-zeitlichen Expressionsmuster von *FGFRa, dof and spry2* zeigen, (a) dass *spry2* und *FGFRa* in den selben Zellen an der Knospenbasis koexprimiert werden und (b) dass *FGFRa* (schwach) und *dof* (stark) koexprimiert werden. Zusammengefasst legen die gezeigten Daten eine Existenz von zwei FGFR Signalwegen nahe. Wie zuvor gezeigt, ist der erste Signalweg an der Knospung beteiligt und aktiviert möglicherweise den FGFRa abhängigen feedbackloop über spry2. Ein zweiter FGFR Signalweg könnte dabei an der Knospe und am Kopf adulter Tiere aktiviert werden – in einem Bereich, in dem die mRNA des docking Proteins Dof stark exprimiert wird und die beiden FGFRs eine schwache Expression aufweisen. Hierbei könnte der FGFR die Migration von Zellen kontrollieren oder modulieren und/oder an der Differenzierung von Zellen für eine funktionierende Kopfstruktur notwendig sein. Eine weitere Untersuchung dieser potentiellen Funktion und deren molekularen Netzwerk in welchem *Hydra* FGFR aktiv ist, ist daher interessant für weiterführende Studien.

Contents

1. Introduction

1.1. Phylum Cnidaria

Cnidaria, a sister group of Bilateria, has relatively simple morphology and complex gene repertoire (Galliot et al. 2009; Steele et al. 2011; Kelava et al. 2015). The phylum Cnidaria encloses ~9000 species with remarkably distinct form and function (Steele et al. 2011). It is an ideal model system for studying developmental and cellular processes such as pattern formation, regeneration, stem cell biology, an evolution of animal embryonic development (David and Murphy 1977; Bode 2003; Holstein et al. 2003; Darling et al. 2005; Bosch 2007; Bode 2009; Bosch 2009; Genikhovich and Technau 2009). Cnidaria comprises two major classes – Anthozoa and Medusozoa. Anthozoa is composed of classes, Hexacorallia and Octocorallia. Likewise, Members of medusozoan class are Hydrozoa, Cubozoa, Staurozoa and Scyphozoa (Collins et al. 2006; Steele et al. 2011; Kelava et al. 2015; Lanna 2015) (Fig. 1).

Characteristically cnidarians are radially symmetric (Finnerty et al. 2004). Along with that they can also exhibit directional asymmetry and even bi-radial symmetry (Dunn and Wagner 2006; Jouiaei et al. 2015). Despite their diversity in size, habitat, toxicity, and morphology, cnidarians possess some common cellular characters, for example, two single layered epithelia, the ectoderm and the endoderm, which are separated by a mesoglea (an extra-cellular matrix), as well as neuromuscular systems (Technau and Steele 2011; Moroz et al. 2014). According to molecular evidence and fossil data, the origin of cnidarians has been estimated to be between 600 million years (discussed up to 750 million years ago prior to the Ediacaran period) and ~550 million years ago prior to the Cambrian, when its major taxa diversified from the remaining metazoans (Park et al. 2011; Steele et al. 2011; Park et al. 2012; Jouiaei et al. 2015). *Hydra,* an important member of the pre-bilaterian phylum Cnidaria, has a significant ability of regeneration and is appropriate for different types of experimental manipulations (Chapman et al. 2010; Galliot 2012).

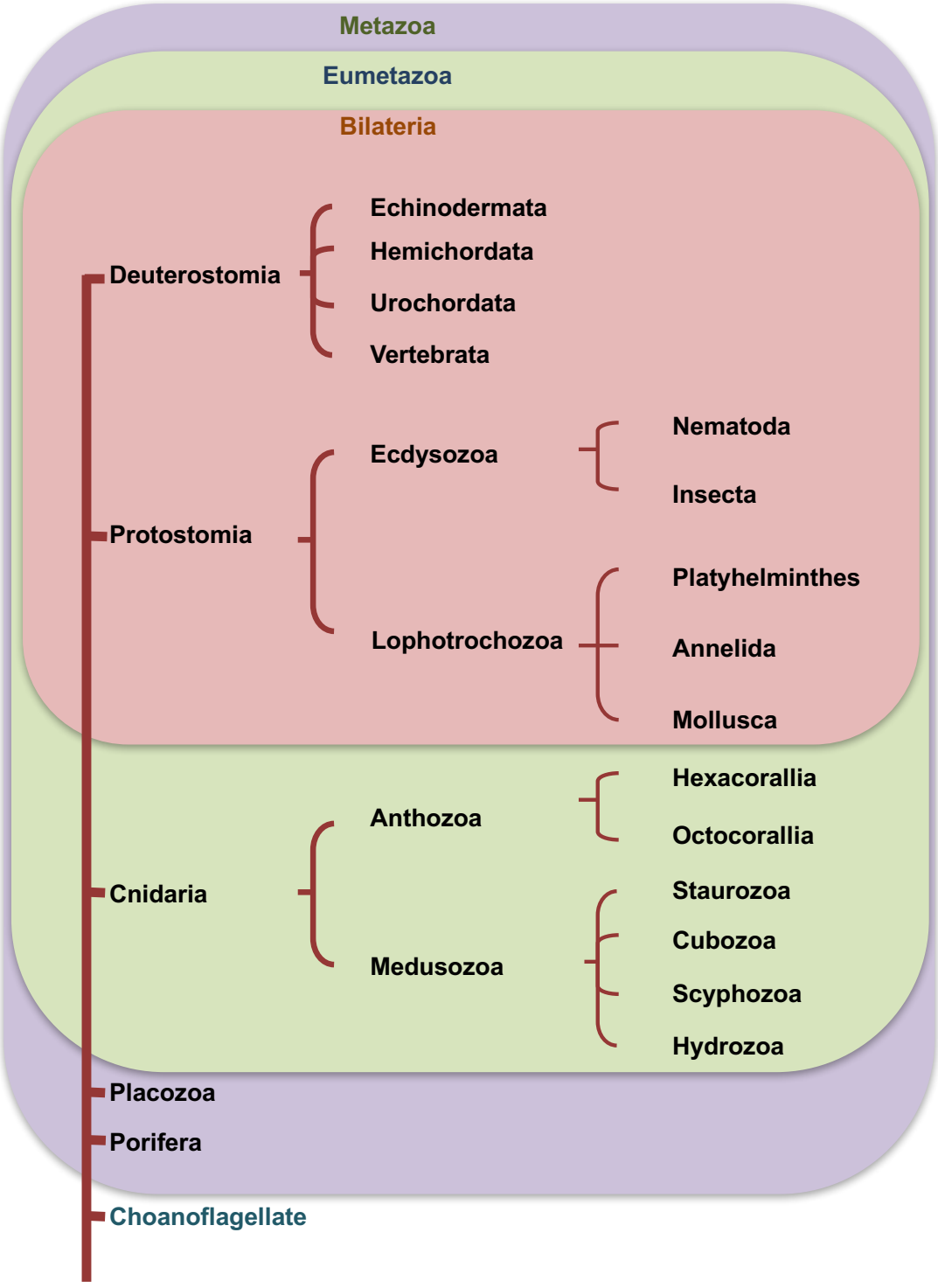

Fig. 1: Graphical representation of the animal evolutionary tree introducing Cnidaria. (Tree does not reflect evolutionary distances)

1.2. *Hydra* – an ideal experimental model organism

Hydra was in detail studied by Trembley in the 18th century (Ratcliff 2004; Galliot 2012). His research was focused on the enormous regenerative capacity of this

fresh water polyp. Despite having a typical feature of plants, i.e. regeneration, he characterized *Hydra* as an animal, considering its nervous and digestive systems. Research of Abraham Trembley promoted *Hydra* as a model system for studying development, morphogenesis, regeneration, axial patterning, stem cell biology, neurogenesis and evolution of regulated developmental genetic programs (Galliot and Schmid 2002; Galliot et al. 2009; Chapman et al. 2010; Galliot 2012; Pierobon 2012). A simple body plan, rapid reproduction time and extreme regenerative potential of *Hydra* polyps make him suitable for being used as a model organism for experimental research. *Hydra* research has contributed addressing numerous biological questions such as the nature of embryogenesis, neurogenesis, induction by organizers, sex reversal, aging, symbiosis, behavior after feeding, light regulation, somatic stem cells multi-potency, temperature-induced cell death and neuronal trans-differentiation (Galliot 2012).

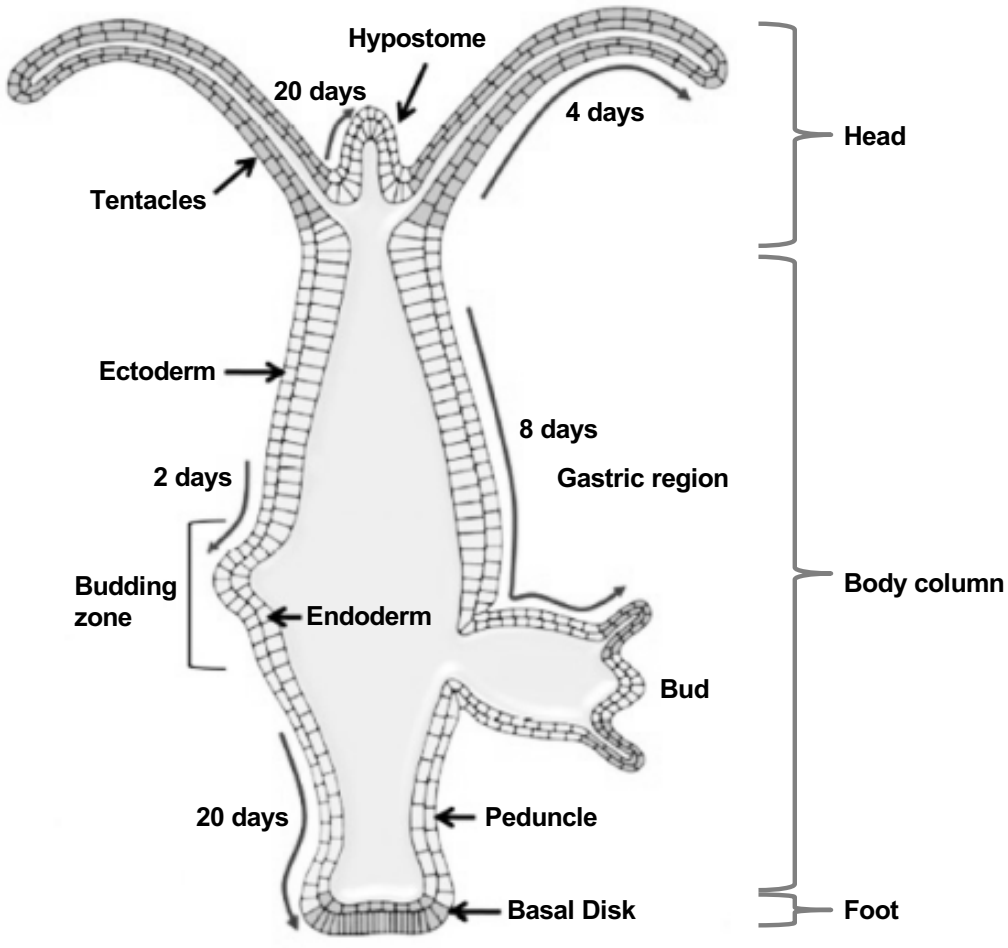

Fig. 2: Schematic longitudinal view of an adult *Hydra* (Bode 2012; Martinez and Bridge 2012). All the regions are labeled. The two lateral protrusions from the body column represent early and late bud stages at the apical and basal ends of the budding zone. Directions of tissue displacement are denoted with arrows.

Hydra is a fresh water polyp, which possesses a simple body plan with only one body axis, an apical head, an intermittent gastric region and a basal disc attaching the individual polyps (Fig. 2). *Hydra* polyps can reproduce sexually and asexually as well, while asexually reproduce through budding via localized unilateral mass tissue movement (Berking 2003; Martinez and Bridge 2012). However, if circumstances are not favorable for particular *Hydra* species such as unusual temperature or starvation, it can reproduce sexually (Nishimiya-Fujisawa and Kobayashi 2012).

1.2.1. Patterning and bud formation in *Hydra*

A properly cultured *Hydra* polyp undergoes asexual growth in the form of budding. A polyp usually sprouts a single bud at one time, which matures and dispatch within about 96 hours (Fig. 3). Mature buds, i.e. a bud with fully formed head and foot region, undergo gradual development. The eight developmental phases are schematically depicted in Table 1.

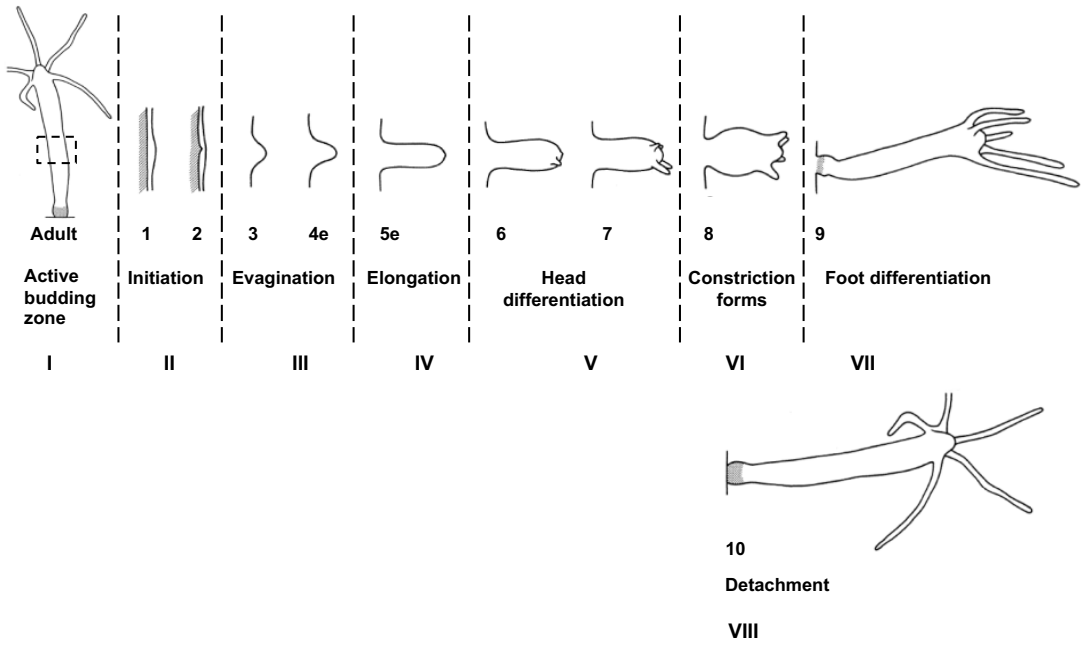

Fig. 3: The budding process in *Hydra*. Developmental phases (I-VIII) and bud stages from 1 to 10 are referred from (Otto and Campbell 1977).

Table 1. *Hydra* bud developmental phases (I-VIII)

I	• **Active budding zone establishment** • Canonical and noncanonical Wnt signaling to establish a budding-competent zone in the gastric column in a species-specific distance from the parent's head (Hobmayer et al. 2000; Guder et al. 2006; Philipp et al. 2009).
II	• **Bud initiation** • Occurs at a single site within the boundary of a budding-competent zone (Martinez et al. 1997; Hassel 1998; Technau and Bode 1999; Hobmayer et al. 2000).
III	• **Evagination** • Competence phase for structure formation. • The evaginating tip increases its positional value and acquires organizer function (Hobmayer et al. 2000; Gee et al. 2010). • PKC activity (Muller 1990; Hassel 1998; Hassel et al. 1998). • Canonical Wnt signaling, PI3-kinase, CREB and MAPK signaling are required (Hobmayer et al. 2000; Fabila et al. 2002; Kaloulis et al. 2004; Manuel et al. 2006; Gee et al. 2010). • Commitment for bud detachment (Sudhop et al. 2004; Münder et al. 2010).
IV	• **Elongation** • Controlled by non-canonical Wnt signaling (Philipp et al. 2009). • Requires planar tissue movements and reorganization of the cytoskeleton. • Tissue recruitment takes place in concentric rings and ceases at stage 6 when the bud becomes autonomous with respect to movement (Otto and Campbell 1977).
V	• **Head differentiation** • Distal differentiation of a hypostome and tentacles. • Canonical Wnt signaling (Hobmayer et al. 2000).

VI	• **Constriction formation** • The proximal decline of the positional value and formation of a circular constriction are interdependent (Bridge et al. 2000; Berking 2003, 2006). • Constriction formation requires the simultaneous decrease of the positional value in ectodermal as well as endodermal cells (Bridge et al. 2000; Berking 2003, 2006). • The constriction starts asymmetrically at the bud base in a position closest to the parent's foot (Bridge et al. 2000). • Cooperation of FGFR, Notch and potentially a noncanonical Wnt pathway (Sudhop et al. 2004; Philipp et al. 2009; Münder et al. 2010) • The activity of FGFR, dpERK and rearrangement of the actin cytoskeleton required (Hasse et al., 2014).
VII	• **Foot differentiation** • For normal morphogenesis, interaction of head and foot-forming systems are required (Muller 1996) • An autoregulatory circuit involving CnNK2 and the pedin/pedibin peptides induce foot differentiation (Grens et al. 1996; Thomsen et al. 2004). • From stage 5 onwards bud head is not needed for foot differentiation.
VIII	• **Detachment** • An autonomous process starts once the bud elongates and also takes place in explants containing adjacent parent tissue. • FGFR signaling initiates detachment process (Sudhop et al. 2004). • Crosstalk of FGFR to Notch allows boundary formation (Sudhop et al. 2004; Münder et al. 2010; Prexl et al. 2011). • Myoactive peptides induce contraction of the sphincter at the bud base and mediate final shedding of the bud (Yum et al. 1998).

1.3. Typical FGFR pathway (fibroblast growth factor receptor)

Studies on FGFR pathway in vertebrate and invertebrate model systems gave valuable insights into its functions in multiple biological processes (Huang and Stern 2005; Thisse and Thisse 2005; Rottinger et al. 2008; Kadam et al. 2009) (Fig. 4). It is an essential signaling pathway which mediates tissue and organ development, maturation, and homeostasis through the regulation of cell proliferation, survival, migration, and angiogenesis (Eswarakumar et al. 2005; Kadam et al. 2009; Korc and Friesel 2009; Turner and Grose 2010; Ang 2015).

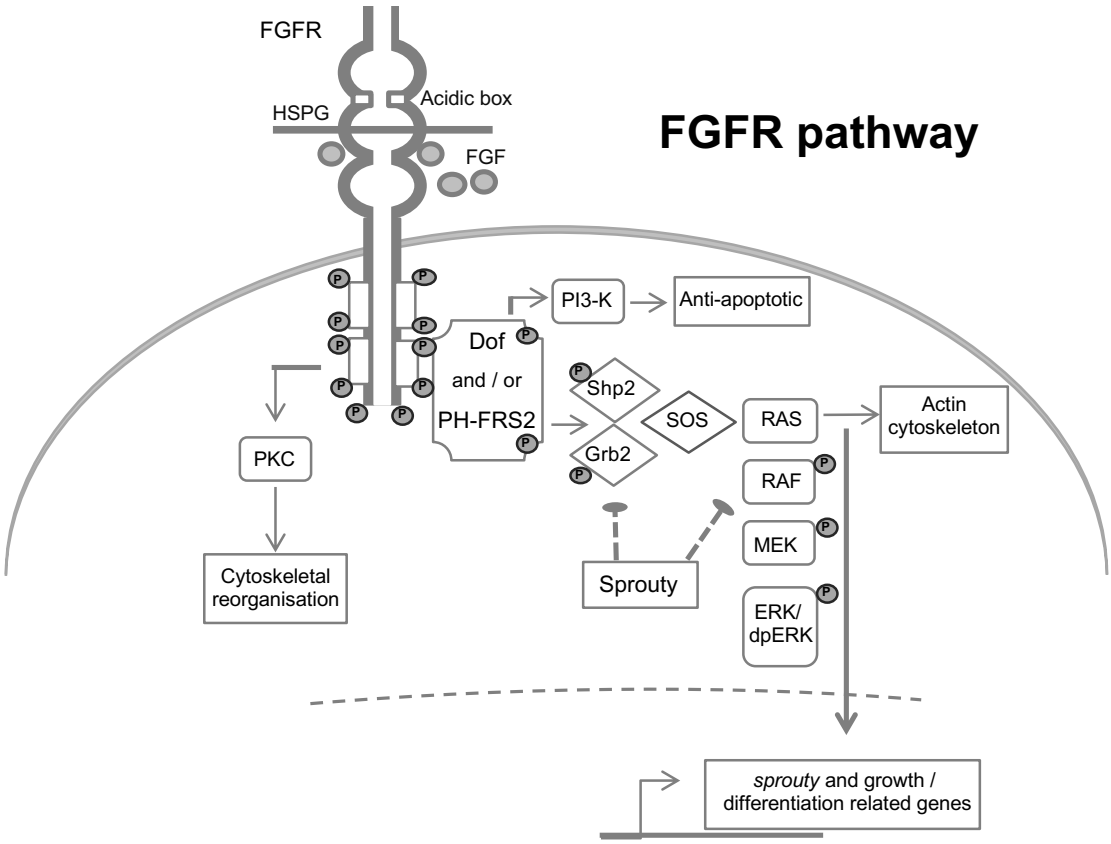

Fig. 4: Schematic representation of three FGFR pathways.

The mammalian FGFR superfamily comprises four RTKs (transmembrane receptor tyrosine kinases) i.e. FGFR1, FGFR2, FGFR3, and FGFR4; along with those it also contains one non-RTK receptor FGFR5 (also known as FGFRL1). In the extracellular ligand-binding domain, the fifth, non-RTK receptor FGFR5/FGFRL1 is homologous to FGFR1–4, but lacks an intracellular tyrosine kinase domain

(Wiedemann and Trueb 2000; Ang 2015; Ornitz and Marie 2015). Domain architecture of FGFR contains an extracellular ligand binding domain, a transmembrane domain, and a cytoplasmic tyrosine kinase domain. The extracellular domain of FGFR comprises a signal peptide at the N-terminus, three Ig-like loops (immunoglobulin-like domains denoted as Ig1-Ig3) with an acidic box present between Ig1 and Ig2, a cell adhesion molecule homology domain, and a positively charged HSPG (Heparan sulfate proteoglycan) heparan-binding domain at the beginning of Ig2 (Schlessinger et al. 2000; Ornitz and Itoh 2001; Wesche et al. 2011). The transmembrane domain connects to the intracellular region, which includes a juxtamembrane domain and two kinase domains which are linked by a tyrosine kinase insert (Mohammadi et al. 2005). The juxtamembrane domain provides binding site(s) for phosphotyrosine-binding domains of proteins such as FRS2 (Desai and Adjei 2016). FRS2 is a lipid-anchored docking protein which upon stimulation by FGF, targets signaling molecules to the plasma membrane (Desai and Adjei 2016).

As mentioned in (Ang 2015; Thisse and Thisse 2005), FGF ligands are stored extracellularly, on the cell surface, and are mainly bound to HSPG of ECM (extracellular matrix). FGFs are released upon enzymatic cleavage and bind to FGFRs with HSPG as a coactivator, resulting in FGFR dimerization and phosphorylation of tyrosine residues in the intracellular domain (Lemmon and Schlessinger 2010; Thisse and Thisse 2005). In vertebrates, consequent recruitment, binding, and phosphorylation of adaptor proteins FRS2, son of sevenless (SOS) and growth factor receptor-bound protein-2 (Grb2) leads to the activation of numerous intracellular signaling cascades. For example, MAPK (mitogen-activated protein kinase), PI3K/Akt (phosphoinositide 3-kinase), JNK (Jun N-terminal kinase), Rho, p38 MAPK and STAT (signal transducer and activator of transcription) (Ang 2015; Thisse and Thisse 2005). Independent of FRS2, binding of the Src homology 2 (SH2) domain of phospholipase C γ (PLCγ) results in the activation of the phosphatidylinositol (PI) cycle and protein kinase C (PKC) (Thisse and Thisse 2005). Specific intracellular signaling cascade activation and associated biologic effects mainly depend on cell type specific variations in FGF ligand and receptor expression, binding specificity for certain adaptor proteins, inter-pathway cross-talk, and the physiologic context (Dailey et al. 2005; Korc and Friesel 2009;

Turner and Grose 2010). FGF receptors differ in their signaling strength and kinetics (Ang 2015). Moreover, Bryant and Stow 2005 confirmed the translocation of FGFR to the nucleus and its active role in transcription, resulting in cell proliferation (Bryant and Stow 2005). Inhibition of FGFR signaling is mediated by tyrosine phosphatases, by Spry and SEF proteins (Tsang and Dawid 2004; Thisse and Thisse 2005). Spry and SEF negatively interact with the receptor itself or any of its downstream signaling elements, also by inducing receptor internalization and degradation via ubiquitination (Tsang and Dawid 2004; Thisse and Thisse 2005; Korc and Friesel 2009; Turner and Grose 2010; Brooks et al. 2012).

1.3.1. Downstream elements of the FGFR pathway: Docking proteins, adaptor proteins, and negative regulators

FGFR signaling through Ras/MAP- kinase and PI3-kinase requires specific docking proteins, Dof in *Drosophila* or FRS2 in vertebrates along with adaptor proteins such as Grb2 and Shp2 (Kouhara et al. 1997; Vincent et al. 1998; Petit et al. 2004; Gotoh 2008). The Ras/MAPK pathway generates negative feedback by activating transcription of the negative regulators Spry and Sef (Lin et al. 2005; Akbulut et al. 2010).

1.3.1.1. Docking proteins

Fibroblast growth factor receptors with their ligands are essential to regulate embryonic as well as adult morphogenesis. Their origin dates back to the common ancestor of Cnidaria and Bilateria (Rebscher et al. 2009; Bertrand et al. 2014; Lange et al. 2014). However little is known about the evolution of the signaling elements used by these receptor tyrosine kinases. In particular, it is not known, whether these ancestral FGFRs require docking proteins. Docking proteins are essential in fly and vertebrates, to specifically transduce the signal of an activated (trans-phosphorylated) FGFR dimer into the cell (Lemmon and Schlessinger 2010). Interestingly *Drosophila* and vertebrates use completely unrelated proteins to fulfill this task. In vertebrates, FRS2 (**F**GF **r**eceptor **s**ubstrate **2**) connects FGFR to PI3 kinase and RAS signaling (Gotoh 2008). FRS2 protein is also termed SNT-1/2 (Suc1-associated neurotrophic factor-induced tyrosine-phosphorylated target) or alternatively, putative FGF-signaling promoter (Guy et al. 2002). In *Drosophila,* Dof (**d**ownstream **o**f **F**GFR) also named as Stumps or

Heartbroken, takes over the function of connecting FGFR to the two pathways (Michelson et al. 1998; Vincent et al. 1998; Imam et al. 1999).

The Dof associated FGFR pathway is well studied in *Drosophila* (Vincent et al. 1998; Dutta et al. 2005; Csiszar et al. 2010; Muha and Muller 2013). Dof contains a Dof-BCAP-BANK domain (DBB), required for receptor binding and binding of ankyrin repeats. This domain represents the most common structural motif required to mediate protein-protein interaction (Wilson et al. 2004). DBB domains share sequence homology with two vertebrate proteins, BCAP and BANK (Battersby et al. 2003). BCAP and BANK proteins are involved in regulation of B-cell receptor specific PI3K activation and calcium mobilization, respectively (Okada et al. 2000; Yokoyama et al. 2002). Posttranslational protein *O*-GlcNAcylation of Dof in an *O*-GlcNAc transferase-dependent fashion is required for its function in the pathway (Mariappa et al. 2011; Muha and Muller 2013). *Drosophila* Dof is expressed exclusively in the region where two FGFRs Heartless (Htl) and Breathless (Btl) are expressed, i.e. in mesodermal, tracheal, and a subset of glial cells (Vincent et al. 1998).

In *Drosophila* FGFR signal transduction, Dof acts downstream of the receptors and upstream of Ras (Wilson et al. 2004). The tyrosine kinase domains of the activated, dimerized FGFR undergo trans-phosphorylation and phosphorylate the adaptor protein Dof. This, in turn, generates Dof binding sites for Grb2 and Csw (homolog of Shp2) and the resulting FGFR signaling complex containing FGFR, FGF ligand, and Dof activates downstream pathways (Vincent et al. 1998; Petit et al. 2004). Dof recruits Csw to the FGFR signaling complex, required for FGF guided cell migration (Petit et al. 2004). Activation of RAS/MAPK signaling is mediated by Grb2, which further recruits the guanine nucleotide exchange factor, SOS. It activates Ras and propagates the signal to the Raf protein kinase, which phosphorylates MEK. This latter kinase activates ERK by double phosphorylation to yield dpERK and finally activates transcription factors (Eswarakumar et al. 2005; Muha and Muller 2013).

FRS2 is the docking protein for FGFR signaling in vertebrates (Kouhara et al. 1997). It comprises of 2 members, FRS2α and FRS2β. For FGF-mediated intracellular signaling, FRS2α acts as a “control center” and also positively

regulates numerous other RTK signaling proteins (Hadari et al. 1998; Gotoh 2008; Gotoh 2009). On the other hand, FRS2β negatively regulates EGFR signaling (Gotoh 2009). Upon tyrosine phosphorylation, FRS2α serves as a platform for the recruitment of multiple signaling proteins, to activate the Ras-MAP kinase signaling cascade (Gotoh et al. 2004). FRS2 proteins belong to the category of IRS related proteins (Kouhara et al. 1997; Guy et al. 2002; Gotoh 2008). They possess an N-terminal myristoylation domain, a phosphotyrosine-binding (PTB) domain and multiple tyrosine phosphorylation sites. The myristoylation domain is used to add lipid anchors and fix the protein in the plasma membrane (Hadari et al. 1998; Gotoh 2008, 2009). The phosphotyrosine binding (PTB) domain is used for binding to FGFRs, and multiple tyrosine phosphorylation sites, which also serve as secondary binding sites for Grb2 and the tyrosine phosphatase Shp2 (Lax et al. 2002). Through SH2 domains, tyrosine- phosphorylated FRS2 binds to the Grb2 adaptor and Shp2 tyrosine phosphatase. Later, several proteins such as SOS, Cbl, and Gab1, constitutively bind to Grb2 via its two SH3 domains (Hadari et al. 1998; Ong et al. 2001; Wong et al. 2002). Recruitment of the Grb2-SOS complex, mediated by FRS2, induces the activation of the Ras/ERK pathway. Cbl acts as an E3 ubiquitin ligase and forms a ternary complex FRS2-Grb2-Cbl with FRS2. This results in ubiquitination and degradation of FRS2 in addition to its receptors. Another ternary complex FRS2-Grb2-Gab1 facilitates tyrosine phosphorylation of Gab1 followed by the recruitment and activation of the PI-3 kinase. In this way, FRS2 brings together both positive and negative signaling proteins to mediate balanced signal transduction. In response to FGF stimulation, the FRS2-Shp2 complex induces tyrosine phosphorylation of Shp2 followed by ERK activation. In the long term, FRS2 serves as a potent ERK activator (Gotoh 2009).

1.3.1.2. Adaptor protein: Growth factor receptor-bound protein-2 (Grb2)

Grb2 is a vital protein in signal transduction (Tari and Lopez-Berestein 2001). It is a ubiquitously expressed adaptor protein, which modulates the extracellular signals during transduction to downstream pathways through its central Src homology 2 (SH2) and the two flanking Src homology 3 (SH3) domains (Radtke et al. 2016). Grb2 play both positive and negative roles in the regulation of RTK

signaling (Belov and Mohammadi 2012). The N-terminal SH3 (N-SH3) is constitutively associated with SOS (mammalian homolog to the *Drosophila* protein) and a guanine nucleotide exchange factor (GEF). The Grb2–SOS complex in non-stimulatory conditions remains in the cytoplasm. Upon receptor activation, phosphorylated receptor tyrosine kinases (RTKs) and their docking proteins such as Dof, FRS2/3, Shc and IRS1-4 provide sites for the Grb2-SH2 domain binding. This leads to recruitment of SOS to the plasma membrane, resulting in the activation of mitogen-activated protein kinase (MAPK) signaling cascade (Ahmed et al. 2015).

Without a canonical Grb2 SH2 domain–binding motif, FGFRs are incapable of recruiting the Grb2-SOS complex directly to the activated receptor (Belov and Mohammadi 2012). FGFRs phosphorylate FRS2α and β, which provide a platform for the Grb2-SOS complex recruitment to the receptor (Bar-Sagi et al. 1993; Kouhara et al. 1997). Furthermore, Grb2 positively regulates FGFR signaling by binding to the phosphatase Shp2. Shp2 then dephosphorylates Spry proteins (FGF signaling inhibitor) (Agazie et al. 2003).

1.3.1.3. Shp2: SH2 domain-containing tyrosine phosphatase 2

SHP2/Shp2 is a ubiquitously expressed non-receptor tyrosine phosphatase which particularly promotes activation of the RAS/MAPK pathway (Dance et al. 2008; Puri and Walker 2016). Shp2 proteins and their invertebrate orthologs are known by various names in different species such as PTPN11/Ptpn11 ((protein-tyrosine phosphatase non-receptor type 11) in mammals, ptpn11a/b in *Danio rerio*, Csw in *Drosophila melanogaster* and ptp-2 in *Caenorhabditis elegans* (Freeman et al. 1992; Perkins et al. 1992; Tajan et al. 2015).

Shp2 comprises a protein tyrosine phosphatase (PTP) domain and two N-terminal Src homology 2 (SH2) domains. A variety of stimuli such as hormones, growth factors, and cytokines can modulate phosphatase activity of Shp2. SH2 domains are required to mediate Shp2 interaction with phosphotyrosine-containing activators such as RTKs and Grb2 (Noguchi et al. 1994; Cunnick et al. 2001; Cunnick et al. 2002; Tajan et al. 2015). The PTP domain has catalytic activity (Neel et al. 2003; Tajan et al. 2015). In its inactive state, one of the N-terminal SH2 domains blocks the access to the PTP domain (Bode et al. 2003; Perrinjaquet et al. 2010; Li et al. 2014). When ligands bind to their respective receptors, SH2 domains are

recruited to specific phosphotyrosine sites on receptors using ligand or receptor-associated adaptor proteins which relieve the PTP domain block. Shp2 is as exceptional phosphatase because it can have either positive or negative regulatory effects on intracellular kinase-mediated signaling pathways. For example, it can increase or decrease PI3/AKT and JAK/Stat signaling activity as well as negatively regulate RhoA activity (Puri and Walker 2016).

The tyrosine phosphatase Shp2 mainly acts by dephosphorylating tyrosine-phosphorylated proteins. This step is essential to limit the signal transmission within a cell. Shp2 activates the MAPK pathway by deactivating upstream inhibitors of Ras (Tajan et al. 2015). Shp2 also functions as a docking protein for the Grb2/SOS complex by directly binding to Grb2 (Bennett et al. 1994; Li et al. 1994; Vogel and Ullrich 1996; Dance et al. 2008; Miura et al. 2013). Specifically, activated Shp2 dephosphorylates and deactivates the Spry protein (a negative regulator of Ras) by excluding the p120RasGAP-RAS inhibitor from signaling complexes and activating Src through dephosphorylation of Src-regulatory proteins (Dance et al. 2008; Tajan et al. 2015). The Shp2-mediated modulation of signaling pathways has been shown to regulate cellular proliferation, differentiation, migration and adhesion in various cell types. Shp2 has thus pleiotropic functions (Tajan et al. 2015; Puri and Walker 2016).

1.3.1.4. Regulators of RTK signaling

There are several possibilities to regulate RTK signaling by targeting negative regulators downstream of RTK signaling (Fiorini et al. 2001). Known negative regulators of FGF signaling are Spry, Sef (Furthauer et al. 2002; Tsang and Dawid 2004; Lin et al. 2005; Thisse and Thisse 2005), MAP kinase phosphatase 3 (MKP3), also called Pyst1 (Eblaghie et al. 2003; Tsang et al. 2004), XFLRT3 (Bottcher et al. 2004; Tsang and Dawid 2004), PEA3 (for polyoma virus enhancer activator 3), and ERM (Gawantka et al. 1998; Munchberg et al. 1999; Kudoh et al. 2001; Thisse and Thisse 2005).

Sprouty (Spry) was originally identified as an antagonist of FGF signaling, which patterns apical branching in *Drosophila* tracheae (Hacohen et al. 1998). In *spry* mutants, excessive FGF signaling induced ectopic airway branches. Spry proteins are inhibitors of RTK signaling in vertebrates and invertebrates (Kim and

Bar-Sagi 2004) because it functions in tracheal and limb (branching) development. It is also essential for patterning of the midbrain and anterior hindbrain, of craniofacial and trunk development - mostly in negative feedback loops used to attenuate or terminate FGFR signaling. Thus Spry proteins exert evolutionarily conserved functions in branching morphogenesis, and in vertebrates as well as in *Drosophila*, restricts the number of branches established in the breathing system and kidney (Masoumi-Moghaddam et al. 2014).

Spry proteins possess four evolutionarily conserved members (Spry1, 2, 3, 4) identified in mammals (Dikic and Giordano 2003). Spry proteins contain conserved domains, a Cbl TKB binding motif (Canonical Casitas B-lineage lymphoma), a tyrosine phosphorylation site, a Raf1-binding domain (RBD) and a Spry domain (Mason et al. 2006; Guy et al. 2009). The cysteine-rich C-terminus of Spry is essential for modulation of cellular migration, proliferation, specific localization and function (Yigzaw et al. 2001; Thisse and Thisse 2005). The N-terminal part of Spry is more divergent except that it carries an invariant tyrosine phosphorylation site, which functions by mediating different protein – protein interactions (Thisse and Thisse 2005).

Spry protein positively and negatively modulates FGFR signaling. It functions as negative regulators of downstream targets of RTK signaling in the Ras/Raf pathway. It may act as a facilitator of FGFR signaling by binding to c-Cbl and prevents c-CBL-mediated ubiquitination and subsequent degradation of activated FGFR (Guy et al. 2003; Cabrita and Christofori 2008; Horowitz and Simons 2008; Guy et al. 2009). Spry acts negatively by two ways: (1) upstream of Ras by docking to the adaptor protein Grb2 via its phosphorylated Tyr55, thus blocking interactions with downstream activators and (2) downstream of Ras by binding to Raf1, thus attenuating or terminating FGFR signaling (Edwin et al. 2009). Gain- and/or loss-of-function studies in mouse, *Xenopus* and *zebrafish* confirmed that Spry functions as an antagonist of FGF signaling (Minowada et al. 1999; Furthauer et al. 2001; Mailleux et al. 2001; Nutt et al. 2001; Furthauer et al. 2004).

1.4. FGFR pathway in *Hydra*

Hydra fibroblast growth factor receptor FGFRa (Kringelchen), belongs to the highly conserved superfamily of FGFR transmembrane receptor tyrosine kinases, which emerged in the common ancestor of Placozoa, Cnidaria, and Bilateria

(Rebscher et al. 2009). *Kringelchen* means “small ring” in German language and refers to its transient expression in a ring of cells after bud detachment (Sudhop et al. 2004). Despite dynamic expression in all stages of budding, Kringelchen function could only be traced up to now for the detachment phase (Sudhop et al. 2004; Hasse et al. 2014). In functional studies, overexpression of FGFR in *Hydra* results in autotomy of the body column, while the dominant negative variant inhibited bud detachment and induced simultaneous sprouting of up to three buds in the circumference.

1.5. Hypothesis and objectives

As elements of an *Hydra* FGFR signaling pathway, FGFRa receptor, FGF ligands (FGFa, FGFe, FGFc, and FGFf) (Sudhop et al. 2004; Hasse et al. 2014; Lange et al. 2014) and downstream elements such as PKC, PI3 Kinase, Ras, MEK, and ERK have been identified (Bosch et al. 1995; Hassel 1998; Hassel et al. 1998; Fabila et al. 2002; Arvizu et al. 2006; Manuel et al. 2006; Hasse et al. 2014) (Fig. 5). However, essential downstream elements such as docking proteins, adaptor proteins and negative regulators needs to be explored.

1.5.1. Do Cnidaria contains docking proteins at all?

In vertebrate and invertebrate FGFR signaling, one of the key elements for transducing a signal from receptor to downstream signaling elements are docking proteins. Since Dof and FRS2 are used in a mutually exclusive manner in fly and vertebrate, the question emerged, whether the two FGFR docking proteins already existed in the genome of early derived invertebrates like Cnidaria.

In *Hydra, FGFRa* (*Kringelchen*) is essential for bud detachment (Sudhop et al. 2004). According to (Hasse et al. 2014), the Ras/MEK/ERK pathway might be a downstream target of the *Hydra* FGFRa. The missing link between *Hydra* FGFRa and RAS/MEK/ERK pathway is docking proteins and adaptor proteins. So we asked the question, whether the *Hydra* genome carries docking and adaptor proteins or not. Presence of the docking and adapter proteins in *Hydra* provokes further questions, how is their evolutionary relationship with other known docking proteins from different taxa, if *Hydra* FGFRa uses docking proteins to target the MAPK pathway.

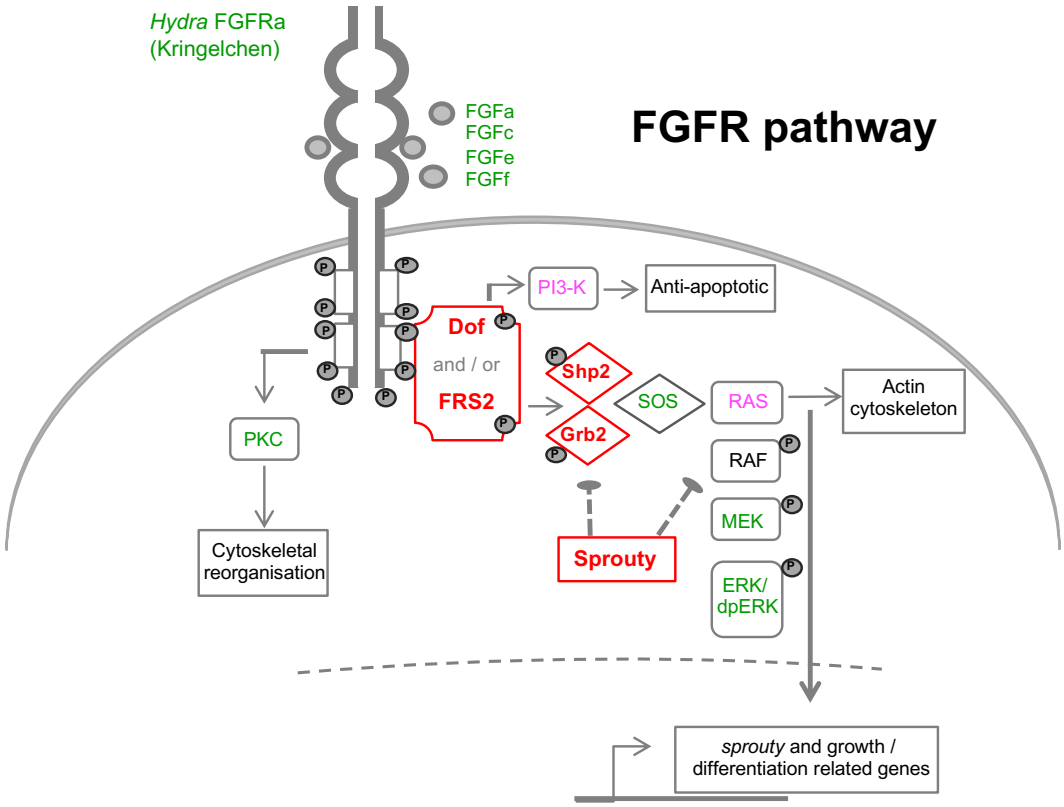

Fig. 5: Putative elements of FGFR signaling in *Hydra* based on the knowledge from fly and vertebrate systems as in Fig. 4. FGFR signaling elements identified by our group are in green letters, elements identified previously by others (pink). Red colored letters indicate unknown downstream elements in *Hydra* FGFR signaling.

1.5.2. Does Spry, a negative regulator of FGFR signaling exist in *Hydra*?

Formation of the limb bud at an existing body stem resembles *Hydra* budding. FGFRs often act in positive or negative feedback loops. It is unknown whether FGFR-dependent autocatalytic loops exist in budding *Hydra*. In vertebrates and invertebrates, negative feedback loops and attenuation of FGFR signaling are established by FRS2- or Dof-mediated MAPK signaling (Kouhara et al. 1997; Vincent et al. 1998; Petit et al. 2004; Gotoh 2008), which results in transcriptional activation of two negative regulators, Sef and Sprouty, (Lin et al. 2005; Akbulut et al. 2010).

Spry, functions as an FGFR signaling antagonist in branching morphogenesis of fly and vertebrates (Hacohen et al. 1998; Guy et al. 2003; Mason et al. 2006; Cabrita and Christofori 2008). A growing bud can be assumed as a “branch” of a *Hydra* polyp, for managing this branch morphogenesis it might need a branch manager. Since the budding process is controlled by FGFR signaling, we can expect a possible recruitment of Spry in the *Hydra* budding management process.

We were interested to know whether the *Hydra* genome encodes one (or more) Sprouties and whether they are expressed in regions, where FGFR signaling is likely. Therefore, another objective of this research was to determine the presence and function of negative regulator Spry in *Hydra*.

1.5.3. Approach to investigate downstream elements in *Hydra* FGFR signaling

The existence of docking proteins and Spry in *Hydra* were investigated with following approach. The available databases were explored for sequences encoding Dof/Stumps/Heartbroken, or FRS2/SNT1, or for their intracellular interaction partners, the downstream adaptor protein Grb2, the tyrosine phosphatase Shp2/Csw, and negative regulator Spry. Database search revealed the conserved sequences of docking proteins, adaptor proteins, and Spry proteins. Predicted proteins were further analyzed for conserved domains, binding sites, and phylogeny. Dof, FRS2, and Spry proteins have deep evolutionary origins and complex phylogenetic distributions. Subsequently, an investigation was performed to reveal the existence of their transcripts and proteins in *Hydra*. Whether the mRNA encoding the docking proteins, *dof* and *FRS2*, as well as the negative regulator *spry* are co-expressed with the *Hydra FGFRa* (*Kringelchen*) were analysed.

2. Results

2.1. Docking proteins

Interference with *Hydra* FGFR signaling revealed the ability of the RTK to activate Ras-MEK-ERK and an unknown pathway controlling F-actin accumulation at the bud base in cells different from the ones accumulating dpERK (Hasse et al. 2014). Since signaling through PI3 K and Ras/MAPK requires docking proteins, the question arose, which of the two known docking proteins of FGFR, Dof in fly and FRS2 in vertebrates, exist in *Hydra*. To address this question, signature domains present in Dof and FRS2 proteins were searched. In Dof, a DBB (Dof-BCAP-BANK) domain and ankyrin repeats are typical (Gotoh 2009; Muha and Muller 2013). The DBB domain is required for activated receptor binding whereas ankyrin repeats mediate protein-protein interactions (Vincent et al. 1998; Battersby et al. 2003; Wilson et al. 2004). In FRS2, the PTB domain (phosphotyrosine binding domain) is required for binding to FGF receptor (Gotoh 2009). Considering those particular domains, *Hydra* EST and protein databases were scanned for homologs of Dof and FRS2. A database search revealed the presence of both the docking proteins encoded in the *Hydra* genome.

2.1.1. Dof, a first candidate FGFR docking protein in Cnidaria

2.1.1.1. Predicted *Hydra* Dof possesses the typical architecture with signature domains

Hydra genomic and EST databases were screened for DBB domain and ankyrin repeats, with reference to the fly Dof protein. A database search revealed EST sequence encoding a *Hydra* Dof homolog (Fig. 6; Fig. S1). Unexpectedly, a Dof-encoding sequence could not be identified in the genome and EST database of *Nematostella*. Anthozoa is considered to be the most ancestral class within Cnidaria (Rentzsch et al. 2008; Steele et al. 2011). Therefore, the presence of Dof in the anthozoan *Acropora digitifera*, the hydrozoan *Hydra* and all other animal genomes searched (supplement Fig. S1), suggests that *Nematostella* has lost the gene. Conserved domain prediction programs predicted following domains in *Hydra* Dof: a DBB domain, an ankyrin repeat domain, and multiple tyrosine's binding sites for proteins such as Grb2, Shp2, PI3K, Src or RasGAP. As well, a common binding site for both, Grb2 and Shp2 was identified. Assembled full-

length *Hydra* Dof protein sequence alignment with vertebrate and invertebrate Dofs revealed the typical and highly conserved DBB domain and ankyrin repeats in *Hydra* Dof (Fig. 6, Fig. S2, S3 and S26).

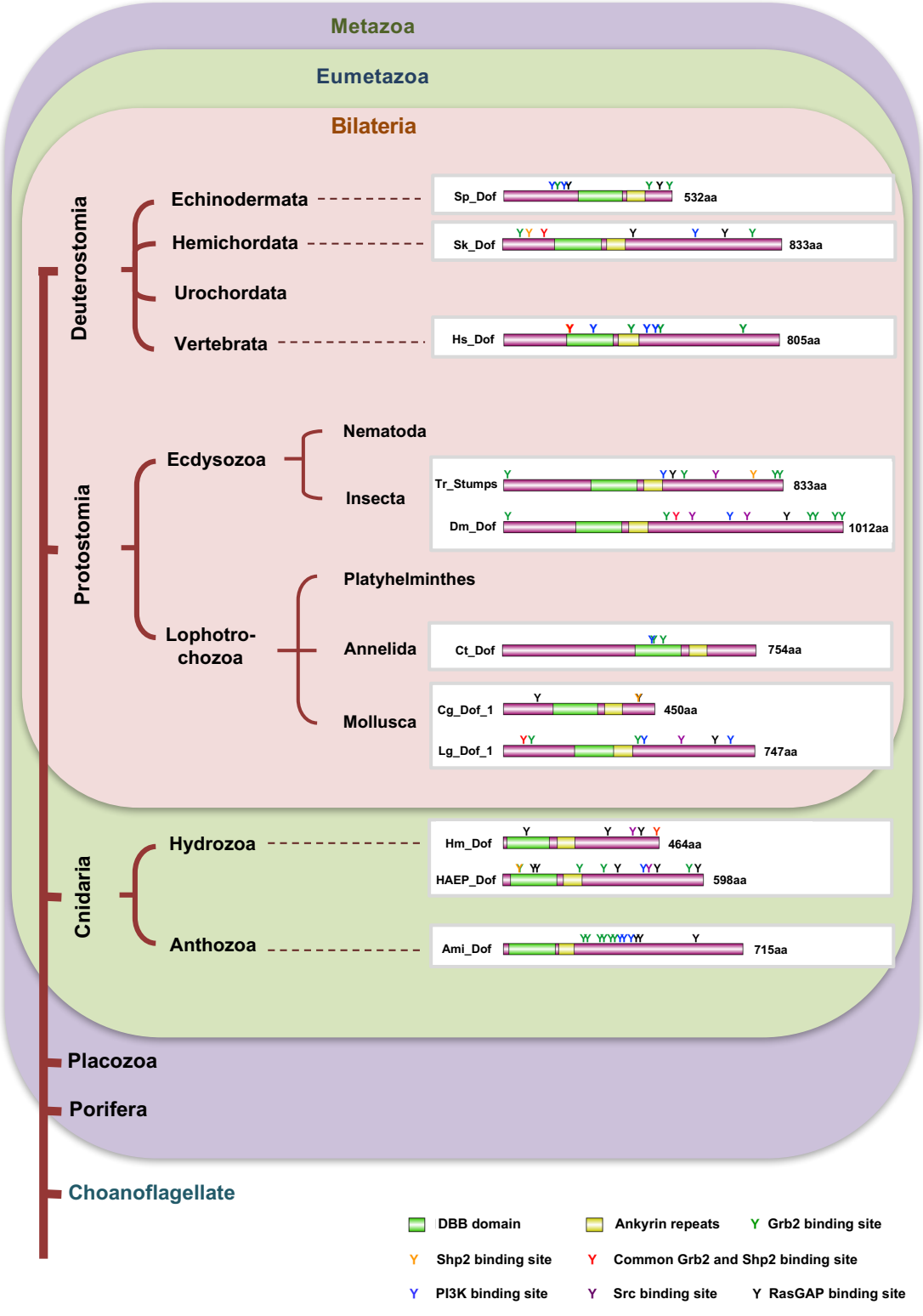

Fig. 6: Schematic summary of Dof domains and binding sites in the evolutionary tree Schematic representation includes DBB motif (green), ankyrin repeats (yellow), common Grb2 and Shp2 binding sites (red colored Y), Grb2 binding sites (green colored Y), Shp2 binding sites (orange colored Y), PI3K binding sites (blue colored Y), Src binding sites (violet colored Y), RasGAP binding sites (black colored Y). Hm_Dof*, Hydra magnipapillata*; HAEP_Dof*, Hydra vulgaris AEP*; Cg_Dof_1, *Crassostrea gigas;* Lg_Dof_1, *Lottia gigantea*; Ct_Dof, *Capitella teleta;* Tr_Stumps, *Tribolium castaneum;* Dm_Dof, *Drosophila melanogaster;* Sk_Dof, *Saccoglossus kowalevskii*; Sp_Dof, *Strongylocentrotus purpuratus;* Hs_Dof, *Homo sapiens.* (For GenBank accession numbers refer to supplement Fig. S1)

Despite the identification of these highly conserved domains in Dof orthologues in *Hydra* and Bilateria, sequences encoding DBB with convincing similarity could not be identified in NCBI gene databases of the placozoan *Trichoplax* and the Choanoflagellates *Monosiga brevicollis,* and *Salpingoeca rosetta*. However, one cannot exclude the possibility of dof in placozoan and Choanoflagellates, as sequence data is not available.

2.1.1.2. Presence of an ancestral Dof in Cnidaria

The next goal was to investigate the evolutionary relationship of *Hydra* Dof with other Dof proteins, using phylogenetic analysis. Phylogenetic relationships were analyzed by considering different outgroups and different associations such as addition or deletion of some species e.g. with highly dynamic genomes. The phylogenetic trees calculated with alternatives outgroup yielded different results.

The Dof proteins contain a Dof-BCAP-BANK domain (DBB), which is required for receptor binding and ankyrin repeats binding. DBB domain represents the most common structural motif required to mediate protein-protein interaction (Wilson 2004). The first aim was to elucidate whether BCAP and BANK domains can tell about the evolutionary relationship among DBB domain containing proteins. Therefore, a tree was drawn using Human BCAP and BANK as outgroup, resulted with an unresolved tree, in which *Hydra* was grouped with insects (Fig. 7 and for sequences refer Fig. S4). This indicates that BCAP and BANK are very likely vertebrate inventions.

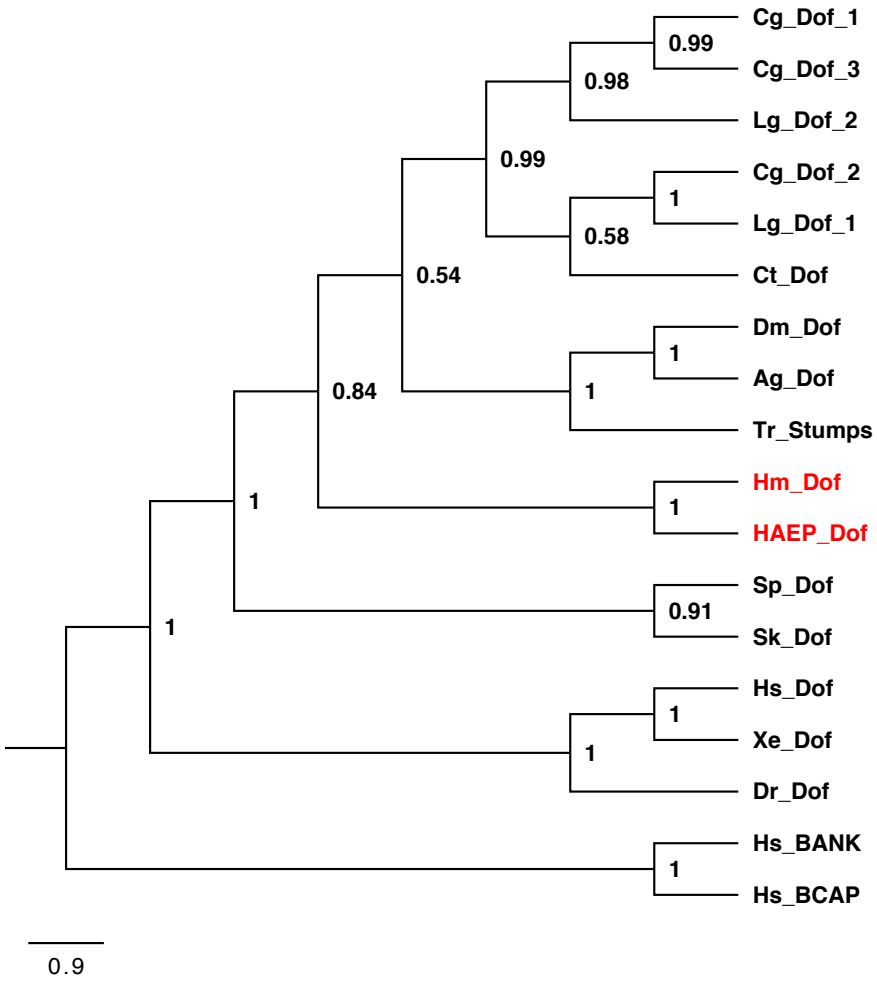

Fig. 7: Phylogenetic tree of Dof proteins rooted with Human BCAP and BANK proteins. Whole protein sequences of Hm_Dof*, Hydra magnipapillata*; HAEP_Dof*, Hydra vulgaris AEP*; Cg_Dof_1, *Crassostrea gigas;* Lg_Dof_1, *Lottia gigantea*; Ct_Dof, *Capitella teleta;* Tr_Stumps, *Tribolium castaneum;* Dm_Dof, *Drosophila melanogaster;* Ag_Dof, *Anopheles gambiae;* Sk_Dof, *Saccoglossus kowalevskii*; Sp_Dof, *Strongylocentrotus purpuratus;* Dr_Dof, *Danio rerio;* Xe_Dof, *Xenopus laevis;* Hs_BCAP*, Homo sapiens* BCAP*;* Hs_BANK*, Homo sapiens* BANK*;* Hs_Dof*, Homo sapiens* were used for phylogeny inference. Hs_BCAP and Hs_BANK protein sequences were used as an outgroup. *Hydra* Dof proteins are marked with red color.

Since Anthozoa is the most ancestral Cnidarian class and well-known species of this phylum i.e. *Acropora millepora* also contains Dof. So, another tree calculated using the putatively ancestral *Acropora millepora* Dof at root. The resultant tree topology does not conform to the currently accepted phylogeny because *Hydra* Dofs grouped within the Ecdysozoa and not within Cnidaria. In this tree, vertebrate Dofs are well separated and molluscs are divided into two groups. However, this tree was not supported with good posterior probability values (Fig. 8).

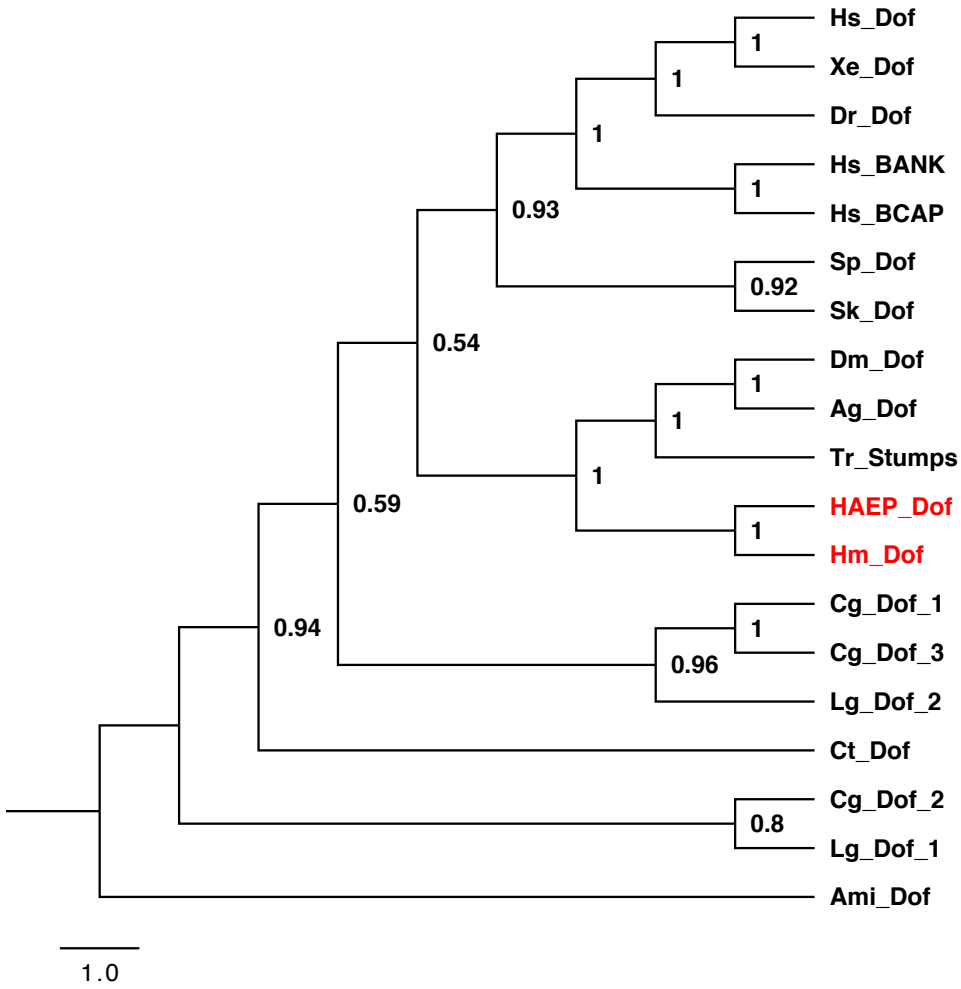

Fig. 8: Phylogenetic tree of Dof proteins rooted with the *Acropora millepora* Dof (Ami_Dof) protein. Whole protein sequences of Hm_Dof*, Hydra magnipapillata*; HAEP_Dof*, Hydra vulgaris AEP*; Cg_Dof_1, *Crassostrea gigas;* Lg_Dof_1, *Lottia gigantea*; Ct_Dof, *Capitella teleta;* Tr_Stumps, *Tribolium castaneum;* Dm_Dof, *Drosophila melanogaster;* Ag_Dof, *Anopheles gambiae;* Sk_Dof, *Saccoglossus kowalevskii*; Sp_Dof, *Strongylocentrotus purpuratus;* Dr_Dof, *Danio rerio;* Xe_Dof, *Xenopus laevis;* Hs_BCAP*, Homo sapiens* BCAP*;* Hs_BANK*, Homo sapiens* BANK*;* Hs_Dof*, Homo sapiens* were used for phylogeny inference. *Acropora millepora* Dof (Ami_Dof) sequences were used as an outgroup. *Hydra* Dof proteins are marked with red color.

For getting a well-resolved tree, a new tree drawn excluding *Acropora* Dof sequence. After deletion of *Acropora* Dof, only ancestral species remaining to root the tree was *Hydra*. Hence the tree calculated with *Hydra* Dof as a root resulted into a well-resolved tree and was supported by acceptable posterior probability values (Fig. 9).

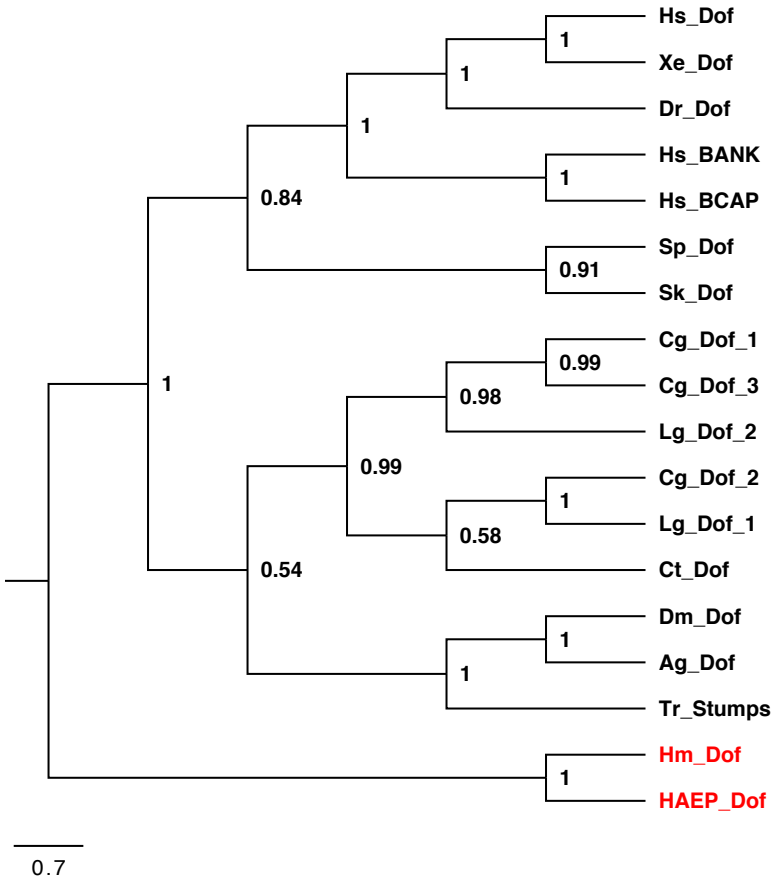

Fig. 9: Phylogenetic tree of Dof proteins rooted with *Hydra* Dof. Whole protein sequences of Hm_Dof*, Hydra magnipapillata*; HAEP_Dof*, Hydra vulgaris AEP*; Cg_Dof_1, *Crassostrea gigas;* Lg_Dof_1, *Lottia gigantea*; Ct_Dof, *Capitella teleta;* Tr_Stumps, *Tribolium castaneum;* Dm_Dof, *Drosophila melanogaster;* Ag_Dof, *Anopheles gambiae;* Sk_Dof, *Saccoglossus kowalevskii*; Sp_Dof, *Strongylocentrotus purpuratus;* Dr_Dof, *Danio rerio;* Xe_Dof, *Xenopus laevis;* Hs_BCAP*, Homo sapiens* BCAP*;* Hs_BANK*, Homo sapiens* BANK*;* Hs_Dof*, Homo sapiens* were used for phylogeny inference. *Hydra* Dof sequences were used as an outgroup. *Hydra* Dof proteins are marked with red color.

Again, the vertebrate group was well resolved and supported by posterior probability values. *Strongylocentrotus* and *Saccoglossus* Dof are placed basal to the vertebrate group, and both were supported with the acceptable 0.91 posterior probability value. This is consistent with the current view that Echinodermata and Hemichordata both have derived from the main line of Deuterostomia earlier than vertebrates. Interestingly, mollusk Dofs are divided into two groups again, with the annelid Dof (*Capitella*) being basal to one of them. The support with 0.99 posterior probability value might indicate an early split within spiralian Dofs and loss of one Dof in *Capitella*. Within Ecdysozoa, Diptera are well separated from

Coleoptera. The tree with *Acropora* Dof sequence as an outgroup does not resolve, therefore it is not possible to use *Acropora* Dof as a root (Fig. 8). So, a tree plotted with *Hydra* Dof as an outgroup generates acceptable phylogeny (Fig. 9).

2.1.1.3. *dof* mRNA is expressed in the bud.

Database analysis confirmed the existence of *dof* in *Hydra*, though its mRNA and protein localization was yet unknown. Therefore, *dof* cDNA was cloned to construct probes for *in situ* hybridization (Fig. S19).

Hydra FGFRa is known for its strong expression during bud development from early stages onwards and weak expression throughout the whole body (Sudhop et al., 2004). Docking proteins for the *Hydra* FGFR are expected to be expressed in regions, where FGFR expression is reported. To test this hypothesis, we first carried out the whole mount *in situ* hybridization with the *Hydra dof* using RNA probes and compared expression domains in the bud referring to the ten bud stages (Otto and Campbell, 1977).

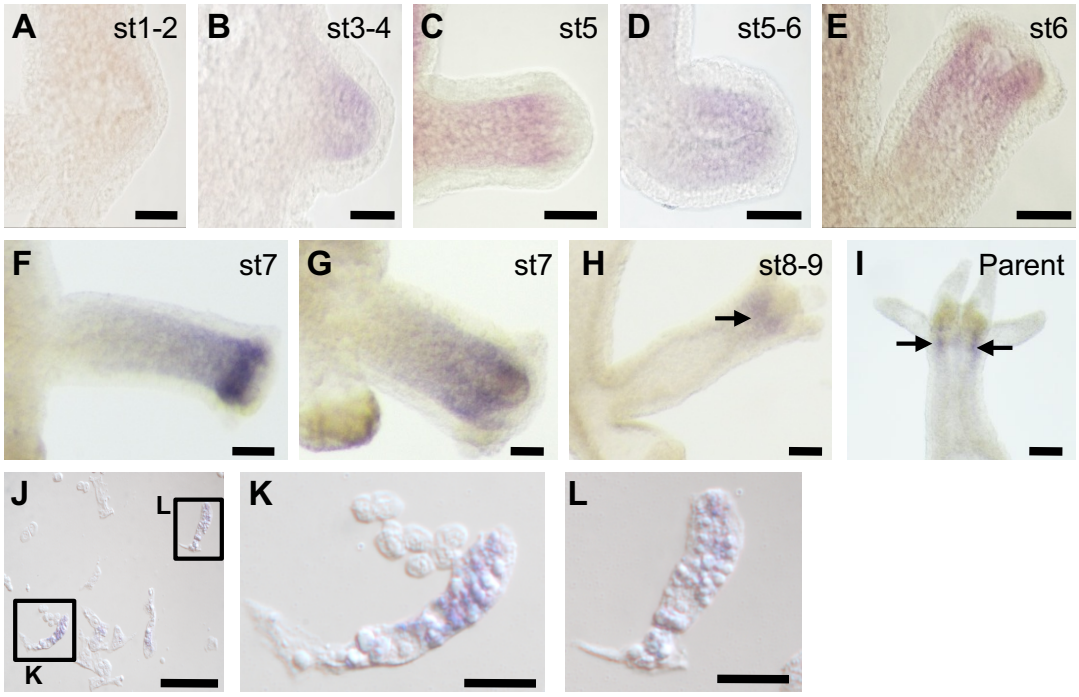

Fig. 10: Expression pattern of *dof* in budding *Hydra vulgaris AEP* polyps and single cells. Expression of *dof* in buds (A-H), in parent head (I) and in single cells preparation (J, K, and L). Scale bar: 100 µm.

In situ hybridization revealed *dof* transcripts from stage 3 onwards in endodermal cells of the growing bud (Fig. 10 B-H). Strong expression all over the bud starts

from stage 3 (Fig. 10 B) and in the following concentrates in cells of the upper bud and at tentacle bases from stages 7 to 9 (Fig. 10 F-H). *dof* mRNA remained detectable weakly close to the parent tentacle base (Fig. 10 I). *dof* expression was restricted to endodermal epithelial cells as revealed by an *in situ* hybridization study with single cell preparations (Fig. 10 J, K, and L)

2.1.2. FRS2, a second candidate FGFR docking protein

An alternative FGFR docking protein, FRS2, is used in vertebrates (Kouhara et al. 1997). Typically, vertebrate FRS2 proteins carry N-terminal myristoylation sites, which ensure the localization of the protein to the plasma membrane (Fig.11). A phosphotyrosine-binding (PTB) domain links it constitutively to FGF receptors. Multiple tyrosine phosphorylation sites are essential to dock downstream signaling proteins, mainly Grb2 and the phosphatase Shp2/csw.

There are no reports about FRS2 being used outside vertebrates to dock FGFR. Nevertheless, the search of invertebrate genomes using the conserved PTB domain uncovered FRS2 proteins in Choanoflagellates, the sponge *Amphimedon queenslandica*, in Placozoa, Platyhelminthes, Mollusca, Annelida, insects and nematodes (Fig. 11 and supplement S1, S5, S6, and S27). Likewise, we found four FRS2 proteins in *Hydra magnipapilata* and one FRS2 protein in *Hydra vulgaris AEP*. The presence of a conserved PTB domain was confirmed by aligning the putative FRS2 proteins from *Hydra* against predicted or verified FRS2 proteins from vertebrates and invertebrates (supplemental Fig. S5).

In vertebrate FRS2 proteins, Grb2 and Shp2 proteins interact with multiple, partially shared, tyrosine phosphorylation sites (Fig. 11). In contrast to vertebrate FRS2, only a few potential tyrosine phosphorylation site's consensus sequences for binding of Grb2 and/or Shp2 adaptor proteins are predicted in the *Hydra* proteins.

Deviating from the vertebrate FRS2 proteins, *Hydra* FRS2 lacks N-terminal myristoylation sites but instead has a PH (pleckstrin homology) domain, which is a sequence feature of proteins involved in signal transduction processes (Haslam et al. 1993; Scheffzek and Welti 2012). Known vertebrate FRS2 proteins do not

contain a PH domain, which analogous to myristoylation may target proteins to the membrane (Delahaye et al. 2000; Uhlik et al. 2005).

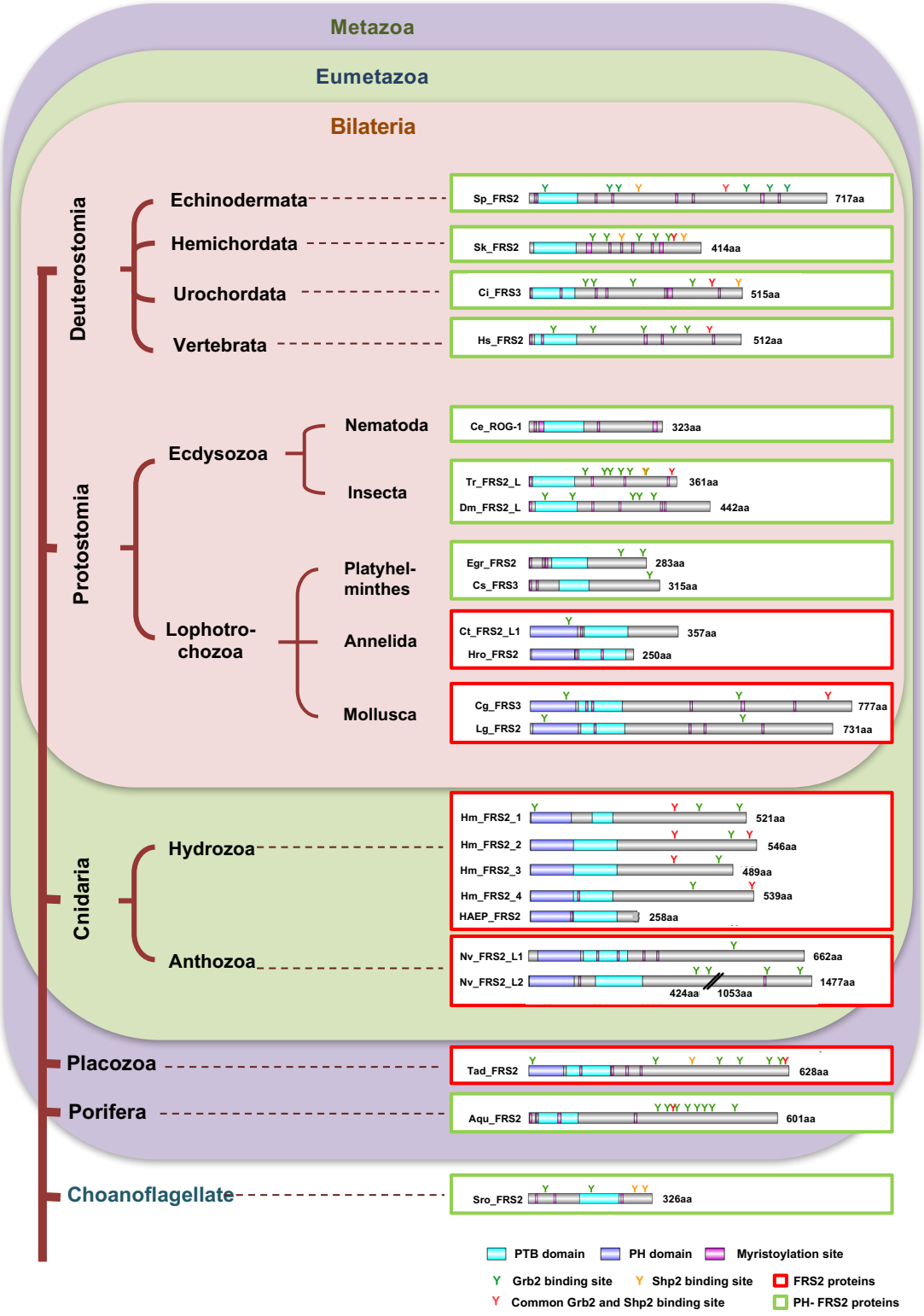

Fig. 11: Schematic summary of FRS2 domains and binding sites in evolutionary tree Includes PTB domain (blue), PH domain (violet), myristoylation site (pink), common Grb2 and Shp2 binding sites (red colored Y), Grb2 binding sites (green colored Y) and Shp2 binding sites (orange colored Y). FRS2 proteins with PH domain

are marked with red color box and FRS2 proteins without PH domain are marked with a green colored box. Hm_FRS2*, Hydra magnipapillata* FRS2; HAEP_FRS2*, Hydra vulgaris AEP* FRS2; Nv_FRS2_L, *Nematostella vectensis* FRS2 like; Sro_FRS2, *Salpingoecia rosetta;* Aqu_FRS2, *Amphimedon queenslandica*; Tad_FRS2, *Trichoplax adhaerens*; Egr_FRS2, *Echinococcus granulosus;* Cg_FRS3, *Crassostrea gigas;* Lg_FRS2, *Lottia gigantea*; Ct_FRS2_L1, *Capitella teleta* FRS2 like*;* Tr_FRS2_L, *Tribolium castaneum;* Dm_FRS2_L, *Drosophila melanogaster* FRS2 like*;* Ce_ROG-1, *Caenorhabditis elegans*; Sp_FRS2, *Strongylocentrotus purpuratus;* Sk_FRS2, *Saccoglossus kowalevskii*; Ci_FRS3, *Ciona intestinalis;* Hs_FRS2, *Homo sapiens.*

Further analysis and sorting of the proteins by their domains, revealed an interesting split of FRS2 proteins into two groups (Fig. 11): The first group displays the domain structure typical for vertebrate FRS2: N-terminal myristoylation site - PTB domain - multiple Grb2 and Shp2/Csw binding sites. The other group lacks the N-terminal myristoylation site and instead carries an N-terminal pleckstrin homology (PH) domain (known to bind phospholipids), an adjacent PTB domain, and only a few Grb2 and Shp2/Csw phosphotyrosine binding sites. To unequivocally distinguish the two FRS2 protein groups, from now on, the first group will be named as “FRS2”, the second one as “PH-FRS2”.

The mutually exclusive distribution of FRS2 and PH-FRS2 proteins is not reflecting the currently accepted relation of animal phyla. FRS2 proteins were detected in Choanoflagellata (*Salpingoeca*), Porifera (*Amphimedon*), Plathelminthes (*Clonorchis* and *Echinococcus*), in the Ecdysozoa (insects and nematodes) and in Deuterostomia (Fig. 11 FRS2 proteins with green boxes). PH-FRS2 proteins, in contrast, were identified in the parazoan *Trichoplax*, in the Cnidaria *Hydra* (Hydrozoa), and *Nematostella* (Anthozoa), and in Spiralia (Mollusca: *Lottia* (Gastropoda), *Crassostrea* (Bivalvia), Annelida *Capitella* (Polychaeta), *Helobdella* (Hirudinea) (Fig. 11 PH-FRS2 proteins with red boxes).

2.1.2.1. Phylogenetic analysis of FRS2 and PH-FRS2 proteins

Despite of using Probcons, T-coffee, ClustalX, Jalview, Gendoc and COBALT programs for alignments, furthermore the use of InterProScan5, NCBI CD search, Pfam and ExpasyProsite programs for conserved domain search and along with MrBayes-3.1.2 and MEGA 5.2 for the calculation of phylogenetic tree, the phylogenetic analysis of conserved domains from both FRS2 and PH-FRS2 proteins resulted in a complicated tree with weak support from posterior

probability values and not conforming to the currently accepted phylogeny of animals (Fig. 12, for alignments and sequences refer to Fig. S1, S5 and S6). The choanoflagellate *Salpingoeca rosetta* FRS2 (Sro_FRS2) and the sponge *Amphimedon queenslandica* FRS2 (Aqu_FRS2) were used as outgroups.

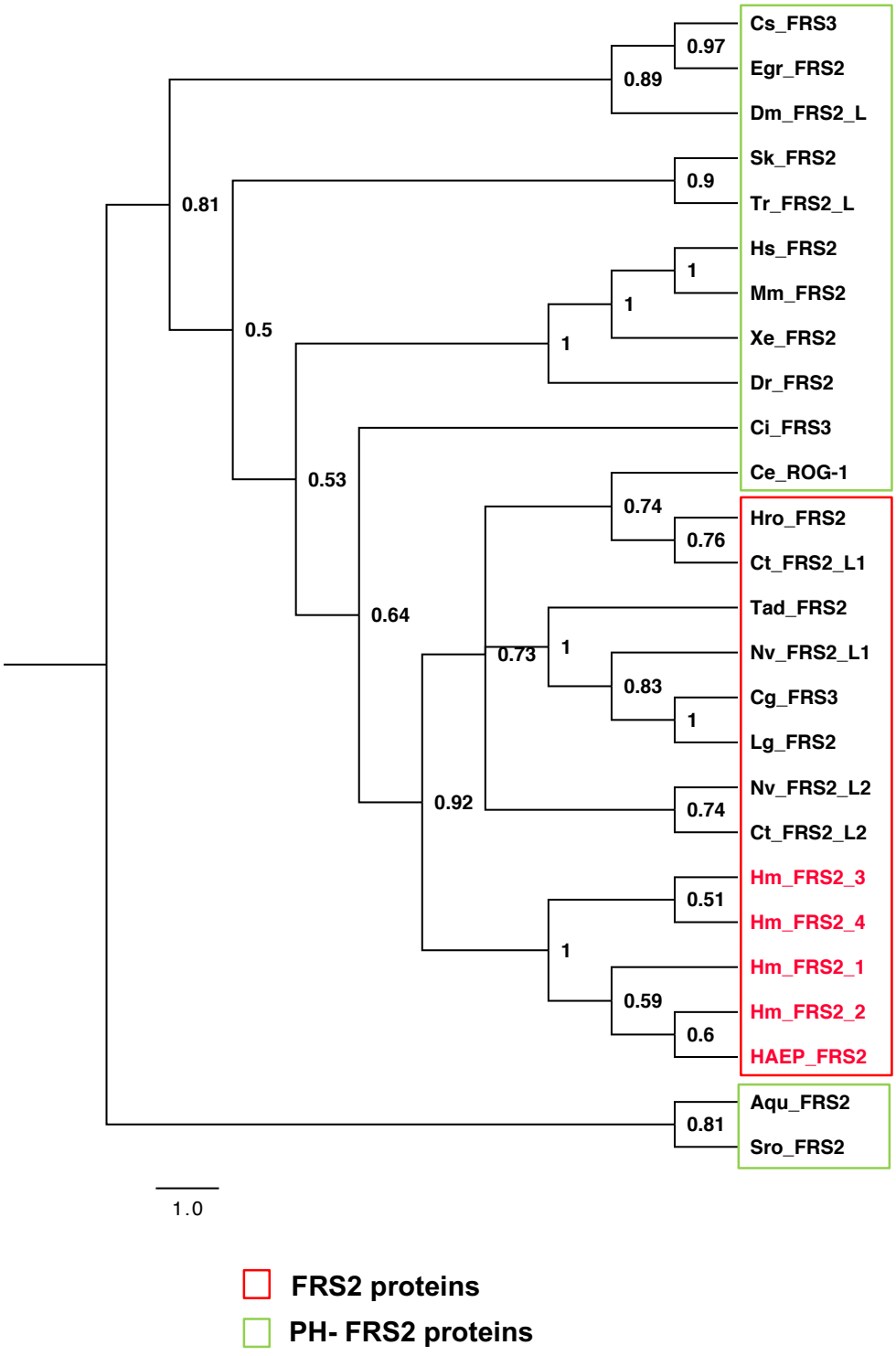

Fig. 12: Phylogenetic analysis of conserved domains in FRS2 and PH-FRS2 protein. A tree was drawn using concatenated PTB domain and myristoylation sites sequences from FRS2, while PTB domain and PH domain sequences from PH-FRS2 proteins. Hm_FRS2*, Hydra magnipapillata* FRS2; HAEP_FRS2*, Hydra vulgaris AEP* FRS2; Nv_FRS2_L, *Nematostella vectensis* FRS2 like; Sro_FRS2, *Salpingoeca rosetta*; Tad_FRS2, *Trichoplax adhaerens*; Egr_FRS2, *Echinococcus granulosus;* Cg_FRS3, *Crassostrea gigas;* Lg_FRS2, *Lottia gigantea*; Ct_FRS2_L1, *Capitella teleta* FRS2 like*;* Ce_ROG-1, *Caenorhabditis elegans*; Tr_FRS2_L, *Tribolium castaneum;* Dm_FRS2_L, *Drosophila melanogaster* FRS2 like*;* Sp_FRS2, *Strongylocentrotus purpuratus;* Sk_FRS2, *Saccoglossus kowalevskii*; Ci_FRS3, *Ciona intestinalis;* Hs_FRS2, *Homo sapiens;* Mm_FRS2, *Mus musculus;* Dr_FRS2, *Danio rerio;* Xe_FRS2, *Xenopus laevis;* Cs_FRS3, *Clonorchis sinensis;* Egr_FRS2, *Echinococcus granulosus;* Hro_FRS2, *Helobdella robusta. Salpingoeca rosetta* FRS2 (Sro_FRS2) and *Amphimedon queenslandica* FRS2 *(*Aqu_FRS2) were used as an outgroup*.*

The tree also divided FRS2 proteins into two, mutually exclusive groups with the fragmented distribution. One group comprises FRS2 proteins with a PH domain i.e. the above, in *Hydra*, newly defined PH-FRS2 proteins, the other comprised the FRS2 proteins without PH domains i.e. FRS2 group. The exception of *C. elegans* ROG-1 protein is acceptable because ROG-1 is a FRS2 like protein (Matsubara et al. 2007). Conspicuous is the split within the Lophotrochozoa, more precise within the Spiralia (Fig. 12), where FRS2 proteins are encoded in the parasitic flat worms *Echinococcus* (Cestoda) and *Clonorchis* (Trematoda), while the mollusk and annelid FRS2 genes encode members of the PH-FRS2 subfamily. To solve this complication, the first step was a calculation of separate trees for FRS2 (Fig. 13 A) and PH-FRS2 (Fig. 13 B) proteins using *Salpingoeca* and *Amphimedon* to root the FRS2 tree and *Trichoplax* to root the PH-FRS2 tree.

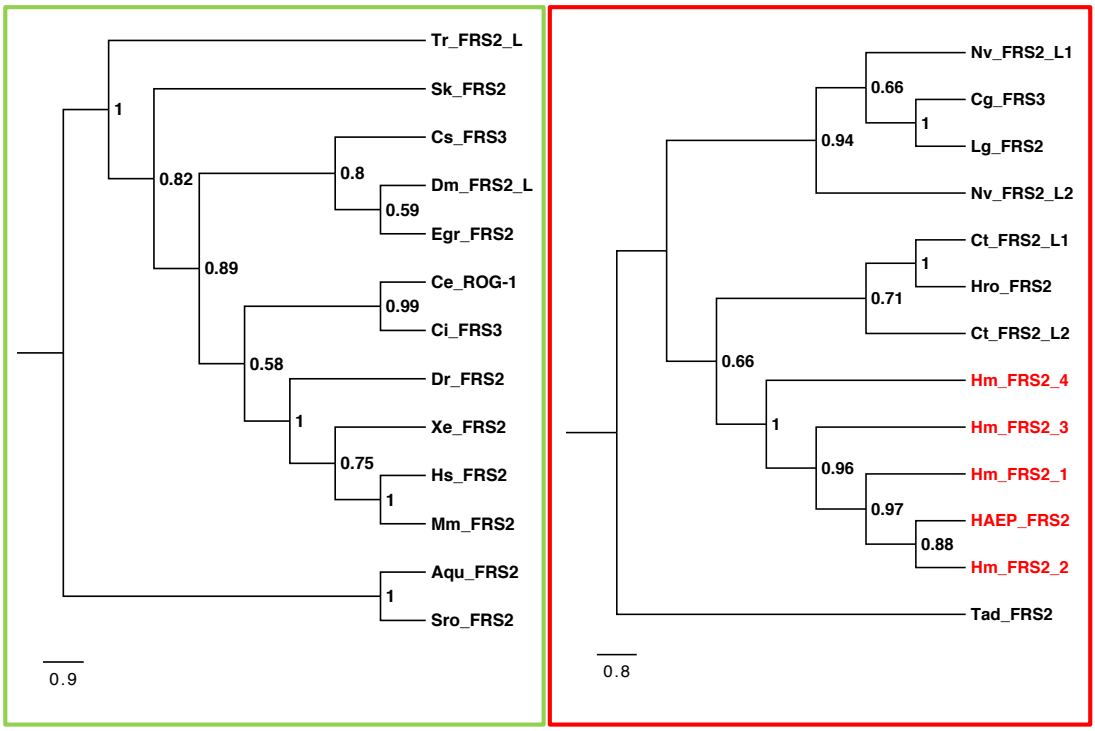

Fig. 13: Phylogenetic analysis of conserved domains in FRS2 (A) and PH-FRS2 proteins (B).

In the FRS2 tree (Fig. 13 A), vertebrates are isolated from invertebrates, but the latter grouped with several unacceptable anomalies: the bug *Tribolium* is placed basal, next to the hemichordate *Saccoglossus*, then a split grouping *Drosophila* with annelid and flatworm and *C. elegans* with *Ciona* and so on. In the PH-FRS2 tree (Fig. 13 B), cnidaria proteins are separated on two sister branches with *Hydra* PH-FRS2 being the sister group to the proteins in annelids (low support) and *Nematostella* PH-FRS2 proteins being a sister group to those of molluscs. The *Nematostella* groups with molluscs and not with *Hydra* is unusual. It suggests that it is difficult to calculate a well resolved FRS2 tree, either because of low sequence conservation, insufficient sequence lengths or even the possibility that the mutually exclusive groups are unrelated. As the tree topology did not conform to the current phylogeny of Metazoa, it was difficult to draw any conclusion from these data so far.

In the search for a solution, the domain structure of FRS2 and PH-FRS2 proteins was reconsidered. PH-FRS2 protein in Placozoa, Cnidaria, Annelida, and Mollusca comprises an N-terminal PH domain, an adjacent PTB domain as well as C-terminal Grb2 and Shp2 phosphotyrosine binding sites. However, they lack

N-terminal myristoylation sites. This domain assemblage resembles two protein families in vertebrates and fly, the so-called insulin receptor substrates (IRS), and the downstream-of-kinase (Dok)-family proteins (also named docking protein or downstream-of-tyrosine kinase) (Yamanashi and Baltimore 1997; Yaffe 2002; Yamasaki and Saito 2004; Boulay et al. 2005; Uhlik et al. 2005; Gotoh 2008; Brummer et al. 2010). Both protein families belong to the membrane-linked docking proteins (MLDP), which are localized in the lipid component of plasma membranes. They are comprised of a membrane-anchored domain of hydrophobic amino acid residues and/or a pleckstrin homology (PH) domain at or close to the N-terminal region and a PTB domain (Huang et al. 2004; Huang et al. 2006; Gotoh 2008). Screening of the *Hydra vulgaris* genome revealed two IRS and one Dok1-like protein (for accession numbers and sequences see supplemental Fig. S7, S8, S9, S10).

The NCBI conserved domain program disclosed a PH and a PTB-FRS2 domain in the annotated *Hydra* FRS2 sequence, although it had initially identified it as a PTB-IRS domain. None of the conserved domain programs directly confirmed *Hydra* FRS2 as FRS2 protein. Since FRS2, IRS, and Dok belong to the IRS-related PTB domain protein category, their PTB domains are similar (Kouhara et al. 1997; Margolis 1999; Margolis et al. 1999; Guy et al. 2002). The programs predicted *Hydra* FRS2-PTB as an IRS-PTB, although BLAST search clearly places it with FRS2 proteins. Since *Hydra* possesses an insulin receptor (Steele et al. 1996), and to decide whether *Hydra* FRS2 is an FRS2 or an IRS protein, FRS2 and IRS proteins were compared. The alignment revealed that most (10/13) of the amino acids needed for the interaction of IRS with insulin receptor are absent in *Hydra* FRS2 (Fig. S11) (Eck et al. 1996; Uhlik et al. 2005). I will, therefore, propose to revise its annotation.

Additionally, the possibility was taken into account that *Hydra* PH-FRS2 proteins are Dok proteins because their domain architecture is similar to a PH and a PTB domain (Yaffe 2002; Uhlik et al. 2005; Gotoh 2008).

To analyze the relationship of FRS2 and PH-FRS2 proteins with MLDPs (Membrane-linked docking proteins) such as IRS and Dok proteins, we aligned FRS2, PH-FRS2, IRS and Dok protein sequences (refer supplement Fig. S12).

We selected only IRS and Dok proteins out of all other MLDPs mentioned in (Gotoh 2008) because IRS and Dok proteins have similar domain architecture to PH-FRS2 proteins. According to several reports (Kouhara et al. 1997; Margolis et al. 1999; Guy et al. 2002), FRS2 and Dok are IRS related proteins and share sequence similarity. The alignment of the *Hydra* FRS2 proteins with the known FRS2, Dok and IRS proteins confirms this argument: the sequences show high similarity in the conserved PH and PTB domains (Supplement Fig. S12). Phylogenetic analysis using the whole sequences (without gaps) revealed five well-resolved protein families plus three isolated branches comprising 6 Dok proteins of parasitic platyhelminths/annelid Doks, two deuterostomian Dok1-L/Dok7, and the nematode ROG-1 protein. All other IRS, Dok4-6, Dok1-3 as well as the PH-FRS2 and FRS2 proteins are clearly separated. The latter two contain sequences of choanoflagellates, sponge (FRS2) and Placozoa (PH-FRS2) seem most ancestral. The tree basis of the five groups is unresolved (Fig. 14 and 15).

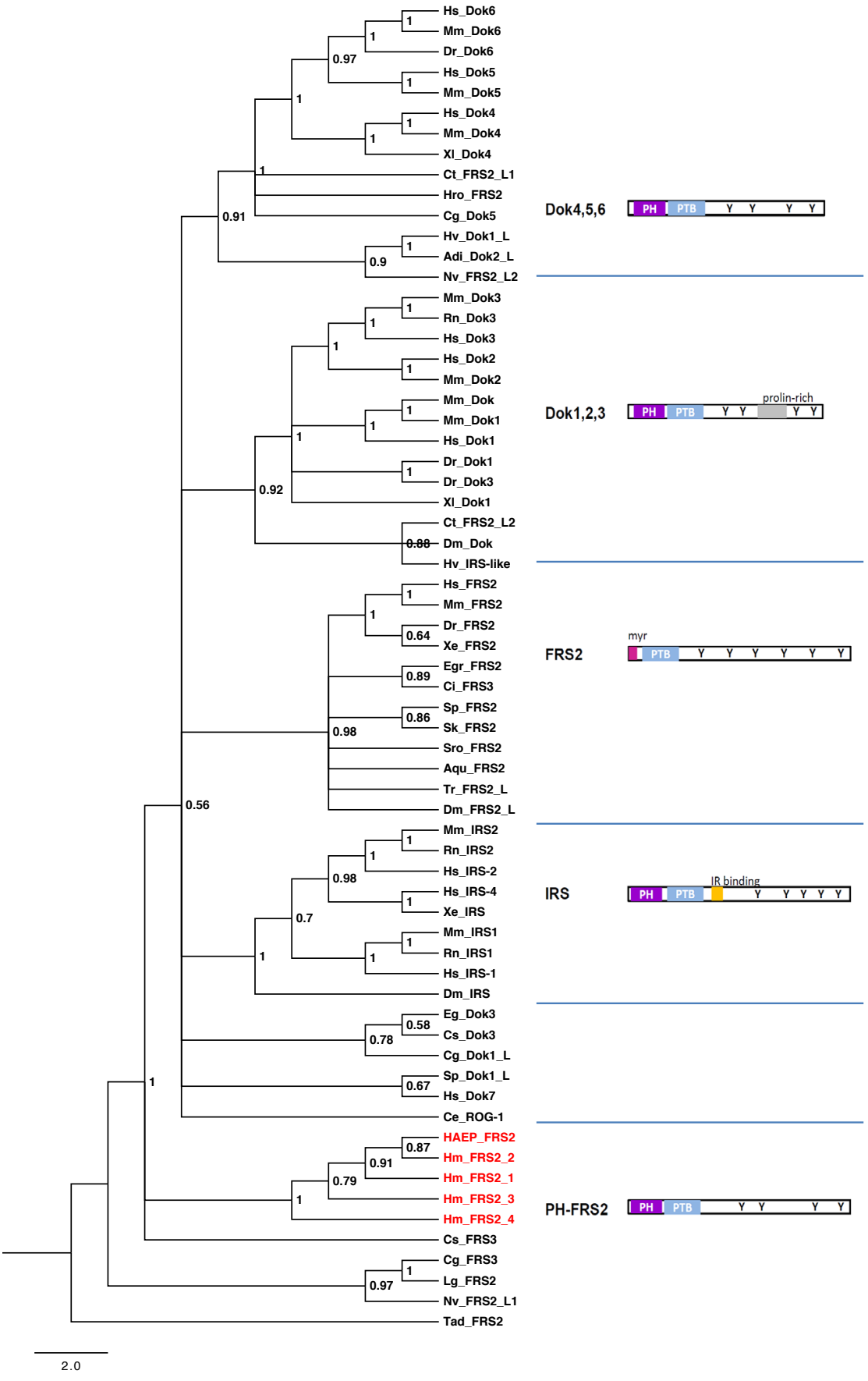
Hs_Dok6
Mm_Dok6
Dr_Dok6
Hs_Dok5
Mm_Dok5
Hs_Dok4
Mm_Dok4
Xl_Dok4
Ct_FRS2_L1
Hro_FRS2
Cg_Dok5
Hv_Dok1_L
Adi_Dok2_L
Nv_FRS2_L2
Mm_Dok3
Rn_Dok3
Hs_Dok3
Hs_Dok2
Mm_Dok2
Mm_Dok
Mm_Dok1
Hs_Dok1
Dr_Dok1
Dr_Dok3
Xl_Dok1
Ct_FRS2_L2
Dm_Dok
Hv_IRS-like
Hs_FRS2
Mm_FRS2
Dr_FRS2
Xe_FRS2
Egr_FRS2
Ci_FRS3
Sp_FRS2
Sk_FRS2
Sro_FRS2
Aqu_FRS2
Tr_FRS2_L
Dm_FRS2_L
Mm_IRS2
Rn_IRS2
Hs_IRS-2
Hs_IRS-4
Xe_IRS
Mm_IRS1
Rn_IRS1
Hs_IRS-1
Dm_IRS
Eg_Dok3
Cs_Dok3
Cg_Dok1_L
Sp_Dok1_L
Hs_Dok7
Ce_ROG-1
HAEP_FRS2
Hm_FRS2_2
Hm_FRS2_1
Hm_FRS2_3
Hm_FRS2_4
Cs_FRS3
Cg_FRS3
Lg_FRS2
Nv_FRS2_L1
Tad_FRS2
0.91
0.97
0.9
0.92
0.88
0.64
0.89
0.86
0.98
0.56
0.7
0.58
0.78
0.67
0.87
0.91
0.79
0.97
2.0
Dok4,5,6
PH
PTB
Y Y
Y Y
Dok1,2,3
prolin-rich
FRS2
myr
IRS
IR binding
PH-FRS2

Fig. 14: Phylogenetic analysis of whole sequences from PH-FRS2 and the membrane - linked docking proteins FRS2, IRS and Dok with their schematic domain structures. Taxa and color code as in Fig. 11. *Hydra* FRS2 proteins are labeled red, and the tree is rooted with *Trichoplax adherens* PH-FRS2. Domain structures of the respective protein families, Dok, IRS, FRS2, and PH-FRS2 are shown on the right. Myristoylation (myr, magenta) of the vertebrate FRS2 N-terminus docks the protein to the membrane. Binding of the pleckstrin homology (PH, violet) domain to phospholipids also serves as a membrane anchor. The phosphotyrosine binding (PTB, blue) domain binds to phosphotyrosines on receptors and downstream signaling elements. Tyrosines (Y) mark potential phosphorylation sites. The proline-rich (gray) region in Dok1 and Dok2 is essential to bind SH3 domain proteins like the adaptor Grb2 or the tyrosine kinase Src. The insulin-binding region of IRS (yellow) is formed by 13 amino acids (labeled red in Fig. S12).

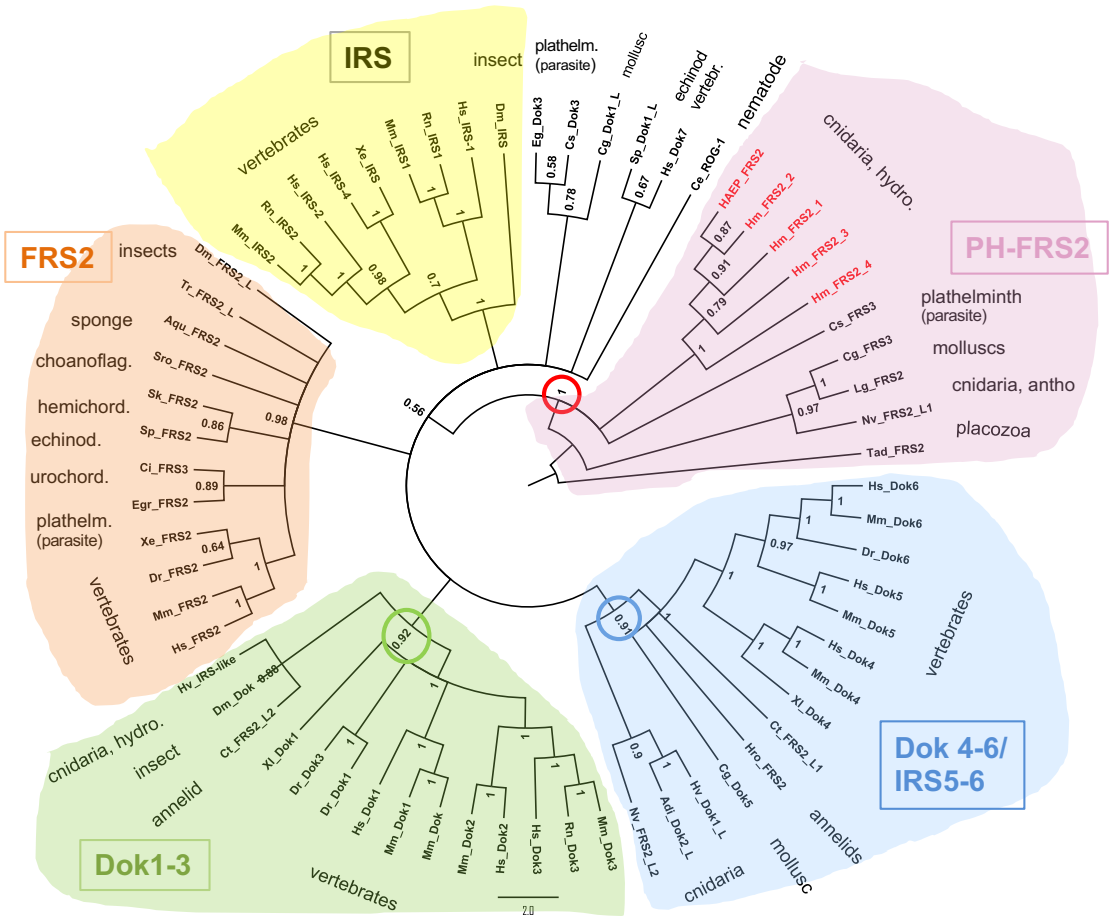

Fig. 15: Phylogenetic analysis of FRS2, PH-FRS2, IRS and Dok proteins. FRS2 (orange color), IRS (yellow color), PH- FRS2 (pink color), Dok 4-6 (blue color) and Dok 1-3 (green color). The tree is rooted with Placozoan *Trichoplax adherens* PH-FRS2.

2.1.2.2. *FRS2*, a weak signal at the tentacle base

It has previously been shown that *Hydra FGFRa* is strongly expressed during bud development and weakly throughout the whole body column (Sudhop et al. 2004). To determine *FRS2* gene localization in *Hydra*, the FRS2 cDNA (566 bp) was cloned (Fig. S19) and a Dig-labelled antisense RNA probe was synthesized for the whole mount *in situ* hybridization. *FRS2* transcripts were detectable weakly at the tentacle base of bud stage 8-9 and parent (Fig. 16 F-H). (Note: Because of my pregnancy, I could not perform this *in situ* hybridization experiment, and my colleague Ellen Lange carried it out. Repetition of the *in situ* hybridization did not improve or alter the result.)

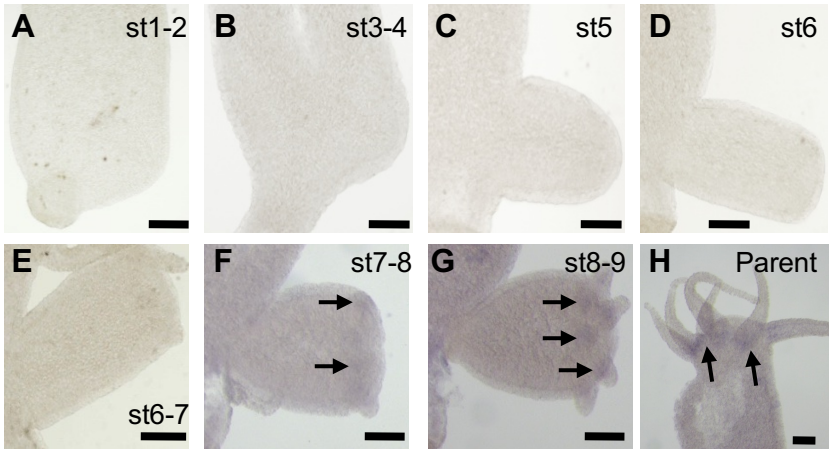

Fig. 16: Expression pattern of *FRS2* in budding *Hydra vulgaris AEP* polyps

2.2. Grb2 protein sequence identified in *Hydra* database

Since docking proteins essential for FGFR signaling clearly exist in *Hydra*, the question arose whether the adaptor protein Grb2 is encoded in the *Hydra* genome, which is required in Bilateria for proper signaling through the FGFR pathway (Belov and Mohammadi 2012).

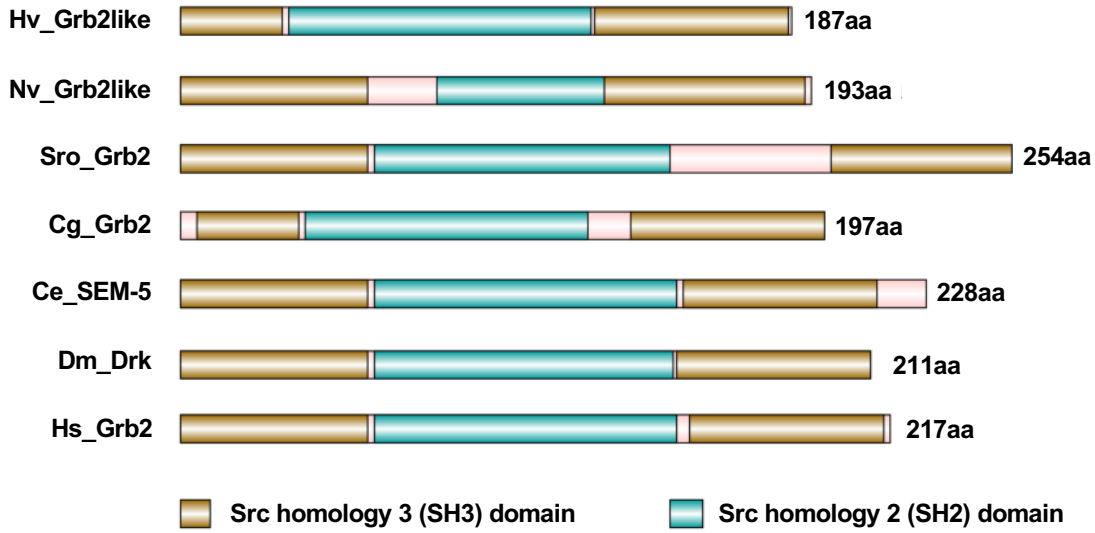

Fig. 17: Schematic summary of Grb2 domains. Src homology 2 (SH2) domain (blue) and Src homology 3 (SH3) domain (brown). Hv_Grb2like*, Hydra vulgaris* Grb2 like; Nv_Grb2like, *Nematostella vectensis* Grb2 like; Sro_Grb2, *Salpingoecia rosetta*; Cg_Grb2, *Crassostrea gigas;* Ce_SEM-5, *Caenorhabditis elegans*; Dm_Drk, *Drosophila melanogaster;* Hs_Grb2, *Homo sapiens.* (See supplement Fig. S13 for GenBank accession numbers and Fig. S28 for sequences.)

By screening *Hydra* databases using known Grb2 proteins as a query, one Grb2-like encoding full-length cDNA sequence was identified in *Hydra vulgaris*. The deduced Grb2 protein has characteristic Grb2 domain architecture (Fig. 17), which is, an SH2 (Src homology region 2) domain flanked by N- and C-terminal SH3 domains.

2.3. Two Shp2 like protein sequences found in *Hydra* database

FGFR signaling requires reduced tyrosine phosphorylation, indicating the need of tyrosine phosphatase. Hence Shp2, i.e. SH2 domain containing tyrosine phosphatase was searched in the database. As mentioned in (Yu et al. 1998; Puri and Walker 2016) Shp2 improves signaling initiated by tyrosine kinases. Shp2 is a ubiquitous protein tyrosine phosphatase (PTP) with a structure and function conserved. Shp2 proteins are known as PTPN11 in mammals, Csw in *D. melanogaster* and ptp-2 in *C. elegans* (Freeman et al. 1992; Tajan et al. 2015).

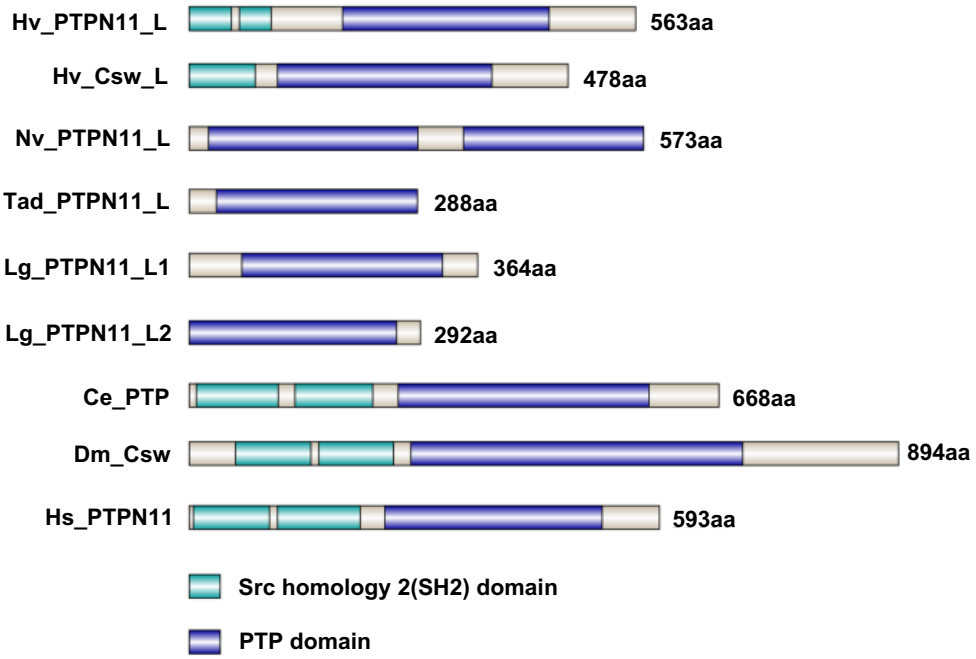

Fig. 18: Schematic summary of Shp2 domains enclose Src homology 2 (SH2) domain (blue) and PTP domain (violet). Hv_PTPN11_L, *Hydra vulgaris* PTPN11 like; Hv_Csw_L, *Hydra vulgaris* Csw like; Nv_PTPN11_L, *Nematostella vectensis* PTPN11 like; Tad_PTPN11_L, *Trichoplax adhaerens*; Lg_PTPN11_L, *Lottia gigantea* PTPN11 like; Ce_PTP, *Caenorhabditis elegans*; Dm_Csw, *Drosophila melanogaster;* Hs_PTPN11, *Homo sapiens.* (See supplement Fig. S13 for GenBank accession numbers and Fig. S29 for sequences)

Databases were screened for the presence of Shp2 in *Hydra* using known Shp2 proteins as query and two Shp2 like proteins were found in *Hydra vulgaris*. The first *Hydra* Shp2 protein, Hv_PTPN11_L, show domain similarity to mammalian PTPN11 protein. The second *Hydra* Shp2 protein, Hv_Csw_L, show domain similarity to invertebrate Csw protein (Fig. 18). Known Shp2 proteins contain two amino-terminal SH2 domains, a catalytic PTP domain and a C- terminal tail with regulatory properties (Jackson et al. 1997; Tajan et al. 2015). The *Hydra* Shp2 proteins contain domain architecture similar to the known Shp2, with the exception of one SH2 domain missing in *Hydra* Csw. *Nematostella, Trichoplax,* and *Lottia* PTPN11 proteins possess only the PTP, but no SH2 domain. This may be because they are “PTPN11 like” proteins.

2.4. Negative regulator Spry

To discover further downstream elements of *Hydra* FGFR signaling the genes encoding the negative regulator Spry was searched. Spry, an intracellular negative regulator of FGFR signaling, attenuates the FGFR signal by interfering with the activity of several intracellular targets (Fig. 5).

2.4.1. Database search revealed four Spry protein encoding sequences in *Hydra*

Databases were screened using the conserved RBD and Spry domains of Spry orthologs (Mason et al. 2006). Sequences encoding four conserved Spry proteins were identified in *Hydra magnipapillata* and termed *Hm_Spry1*, *Hm_Spry2*, *Hm_Spry3* and *Hm_Spry4* (Fig. 19, for accession numbers, see Fig. S14 and for experimental approach Fig. S15). The Spry domain was predicted in all four *Hydra* Spry-encoding sequences concerning conserved domain prediction programs (NCBI CD search, Pfam, ExpasyProsite) (Fig. 19). No Spry sequences could be identified in sponge and Choanoflagellate databases.

Multiple sequence alignments of the *Hydra* Spry proteins with Sprouties of other cnidarian, protostomian, and deuterostomian taxa revealed the presence of all typical conserved domains (Fig. S16): A Cbl TKB binding motif with a central tyrosine phosphorylation site, the Spry domain and the Raf1-binding domain (RBD) (Fig. 19).

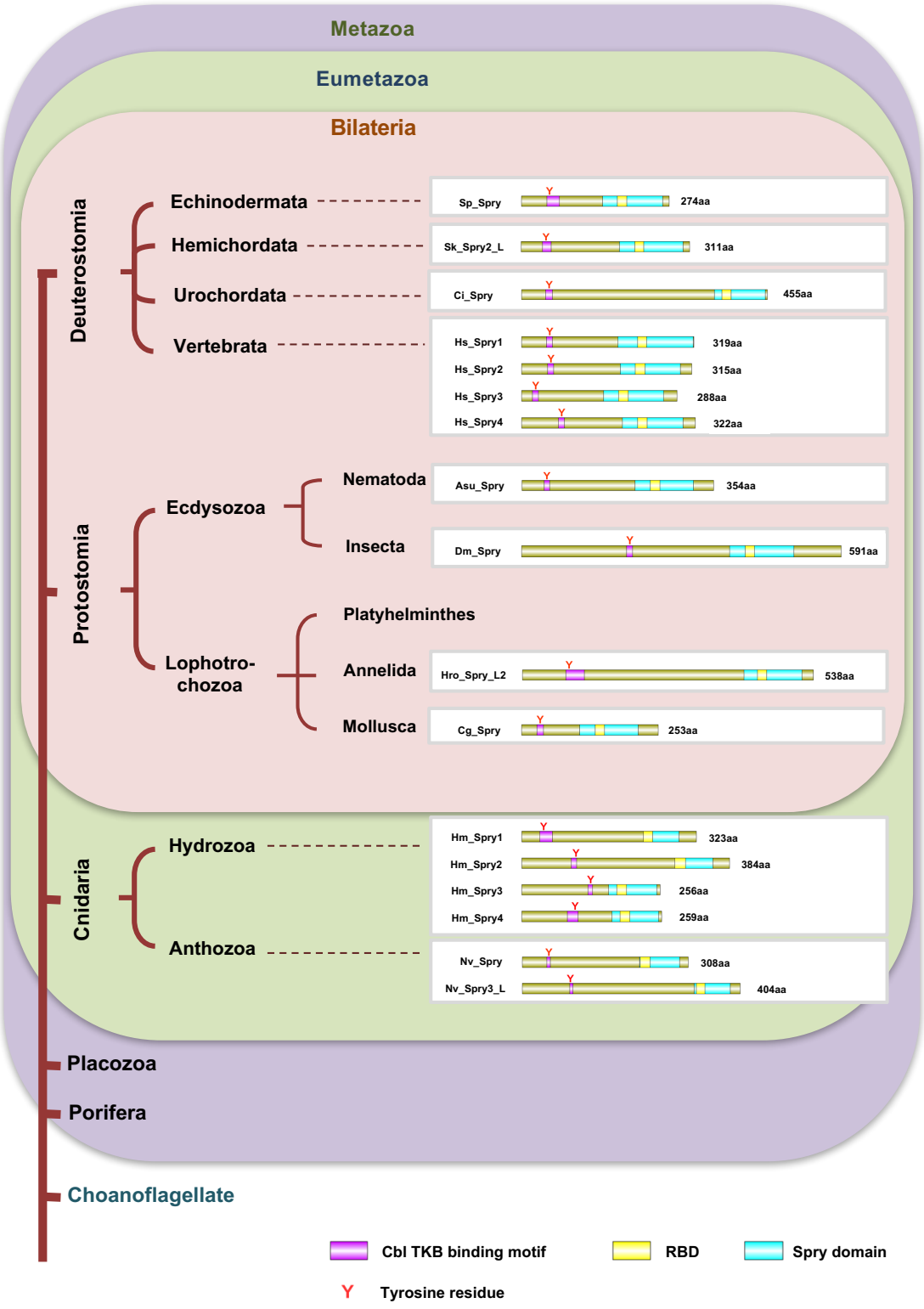

Fig. 19: Schematic summary of Spry domains and binding sites along the evolutionary tree of animals includes Raf-binding domain (RBD, yellow), Spry domain (blue), typical tyrosine residue (red colored Y) and Cbl TKB binding motif (violet). Hm_Spry, *Hydra magnipapillata*; Nv_Spry, *Nematostella vectensis;* Dm_Spry, *Drosophila melanogaster;* Cg_Spry, *Crassostrea gigas;* Hro_Spry_L, *Helobdella robusta* Spry like; Asu_Spry, *Ascaris suum;* Sk_Spry2_L, *Saccoglossus kowalevskii* Spry2

like; Sp_Spry, *Strongylocentrotus purpuratus;* Ci_Spry, *Ciona intestinalis;* Hs_Spry, *Homo sapiens* (For GenBank accession numbers see supplement Fig. S14).

2.4.2. Cnidarian Sprouties are the most ancestral ones

Phylogenetic tree constructed using full length Spry protein sequences revealed the presence of an ancestral Spry in Cnidaria (Fig. 20). Another tree computed using concatenated conserved domain (RBD + Spry domain) protein sequences showed the same (refer supplement Fig. S18).

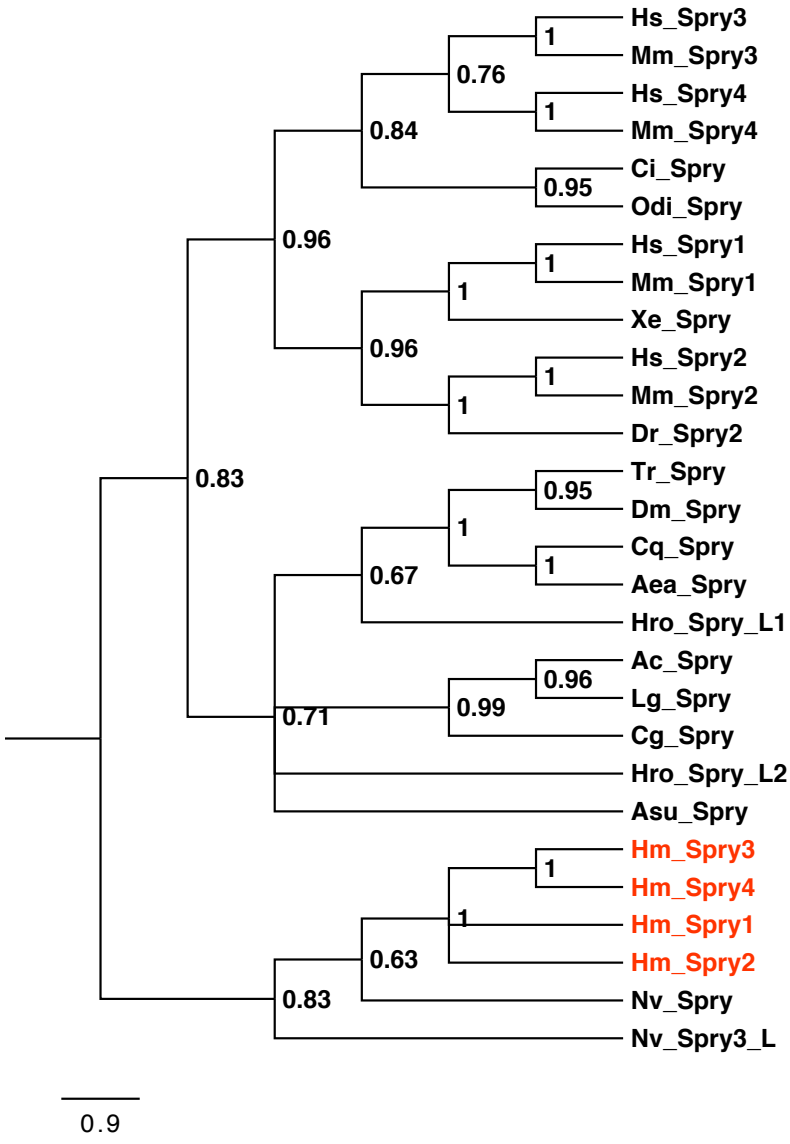

Fig. 20: Phylogenetic analysis of the full length Spry proteins. A tree was generated using whole sequences from Hm_Spry, *Hydra magnipapillata*; Nv_Spry, *Nematostella vectensis;* Dm_Spry, *Drosophila melanogaster;* Tr_Spry, *Tribolium castaneum;* Asu_Spry, *Ascaris suum;* Cg_Spry, *Crassostrea gigas;* Lg_Spry, *Lottia gigantea;* Ac_Spry, *Aplysia californica;* Hro_Spry_L, *Helobdella robusta* Spry like; Cq_Spry, *Culex quinquefasciatus;* Aea_Spry, *Aedes aegypti;* Ci_Spry, *Ciona*

intestinalis; Odi_Spry, *Oikopleura dioica;* Hs_Spry, *Homo sapiens;* Mm_Spry, *Mus musculus;* Xe_Spry, *Xenopus laevis;* Dr_Spry2, *Danio rerio* were used for phylogeny inference. Numbers on branches indicates Bayesian inference posterior probabilities (support values).

The tree topology is partially in agreement with the current view of animal phylogeny, because of some exceptions such as *Helobdella* Spry (Hro_Spry_L2) and *Ascaris* Spry (Asu_Spry) are not well resolved. Deuterostomian Spry are separated from ecdysozoan and lophotrochozoan Sprys with a posterior probability value of 83. Our data thus suggest the first appearance of Spry in the last common ancestor of Bilateria i.e. Cnidaria or a loss in the earlier derived taxa.

2.4.3. Spry2 expression in *Hydra*

Since several Sprouties exist in *Hydra*, the question arose where they are transcribed and what their roles might be. To address the first question, all four *spry* cDNAs were isolated by PCR using specific primers against available EST sequences for *spry1* (310 bp), *spry2* (423 bp), *spry3* (447 bp) and *spry4* (729 bp) (Fig. S19).

2.4.3.1. *spry2* transcript expresses at the bud base

Next, the expression patterns of four *Hydra vulgaris AEP sprys* were analyzed. For *spry1* and *spry4* expression for both sense and antisense probes were found almost in whole body column as well as close to the bud base (Fig. S20). The presence of an endogenous antisense RNA sequence was analyzed with the help of a mRNA Northern blot, and in fact, evidence was found for an endogenous antisense RNA for *spry1* (Fig. S21 and S22). *spry3* mRNA was initially detected in the parent foot endoderm (Fig. S23), but for unknown reasons, this result could not be confirmed later (Fig. S23).

Spry2 mRNA expression was reproducibly found upregulated at the bud base (Fig. 21), an antisense signal was not detectable (not shown). Referring to the ten bud stages (Otto and Campbell 1977), the earliest sign of *spry2* mRNA expression was detected in stage 5 (Fig. 21 B1). Only a few endodermal cells of the stage 5 bud base expressed the gene (Fig. 21 B1 and close-up in B2). From stage 5 onwards, mRNA expression extended circumferentially until *spry2*-positive cells covered the whole bud base in stage 7 (Fig. 21 B1 - D1, B2 - D2).

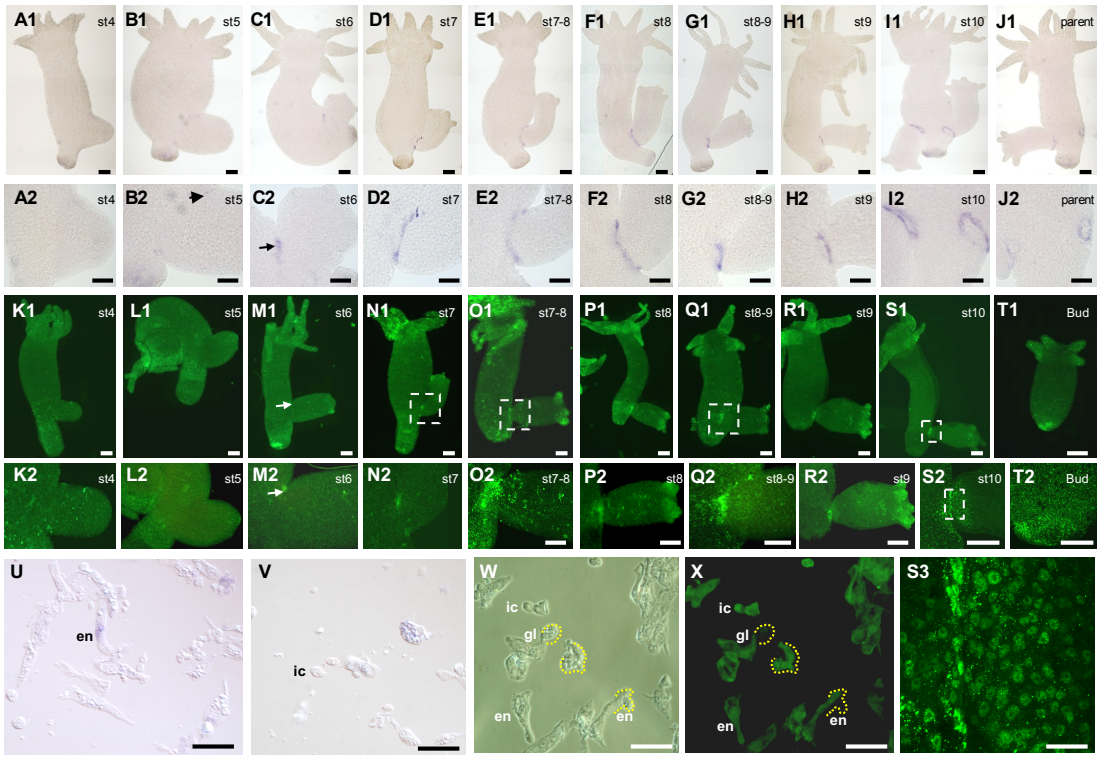

Fig. 21: Expression pattern of Spry2 mRNA and protein distribution in budding *Hydra vulgaris AEP* polyps. (A1-J2) *spry2* mRNA expression, (K1- T2) Spry2 protein expression as detected by anti-*Hydra* Spry2 immunohistochemistry. Bud staging according to Otto and Campbell, 1977. (A1-J1) and (K1- T1) are overviews of budding stages 4 to 10 and the parent animal. (A2-J2) and (K2- T2) are close-ups of (A1-J1) and (K1- T1). S3 is a close up of S2. Expression of *spry2* mRNA in endodermal epithelial cells (en) (U) and protein in endodermal epithelial cells (en) and interstitial cells (ic), except gland cells (gl) (W and X). Dotted yellow line indicates the location of cells. Scale bar =100 µm.

From stage 7 onwards, the strength of mRNA expression increased at the bud-parent boundary (Fig. 21 E1 - J1, E2 - J2). Following bud detachment, a ring of *spry2* mRNA expressing cells persisted at the detachment site in the parent (Fig. 21 J1 and close-up in J2). *In situ* hybridization of single cell preparation was carried out under my supervision by an MSc student, Jens Heller. It revealed expression of *spry2* in endodermal epithelial cells (Fig. 21 U and V).

2.4.3.2. Spry2 protein expresses at the bud base

2.4.3.2.1. Epitope prediction to generate distinctive *Hydra* Spry2 antibody

To obtain a specific *Hydra* Spry2 antibody, the three-dimensional structure of the putative *Hydra* Spry2 (Hm_Spry2) and human Spry2 protein was modeled by the

i-Tasser software (Roy et al. 2010) (Fig. 22), which allowed predicting antigenicity sites for the design of specific anti-peptide antibodies.

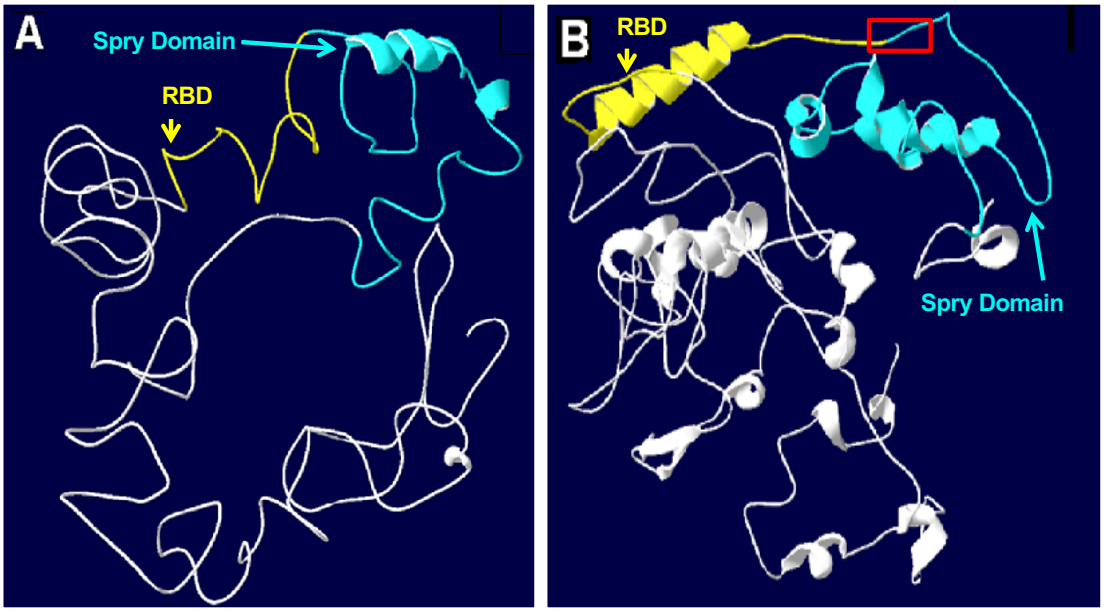

Fig. 22: Predicted three-dimensional structures of (A) human Spry2 and (B) *Hydra* Spry2 proteins. In the three-dimensional structures predicted from i-Tasser software, the RBD domain (yellow) and the Spry domain (blue). The red box indicates the region used as an epitope to generate an anti-Spry2 antibody.

The RBD and Spry domains are essential for Spry activity. The 3D structure indicates the presence of both domains on the protein surface. They thus should be accessible for an antibody directed against a linear epitope. An anti- peptide antibody (red box) was raised using the epitope predicted from 3D structure of *Hydra* Spry2 (refer to Fig. S24 for antibody information and sequence).

2.4.3.2.2. The Anti-Spry2 antibody detected a protein at ~ 43 kDa

Two Anti-Spry2 antibodies were raised commercially against two adjacent peptide epitopes of Spry2 and in two rabbits each (see epitope sequences in supplement Fig. S24).

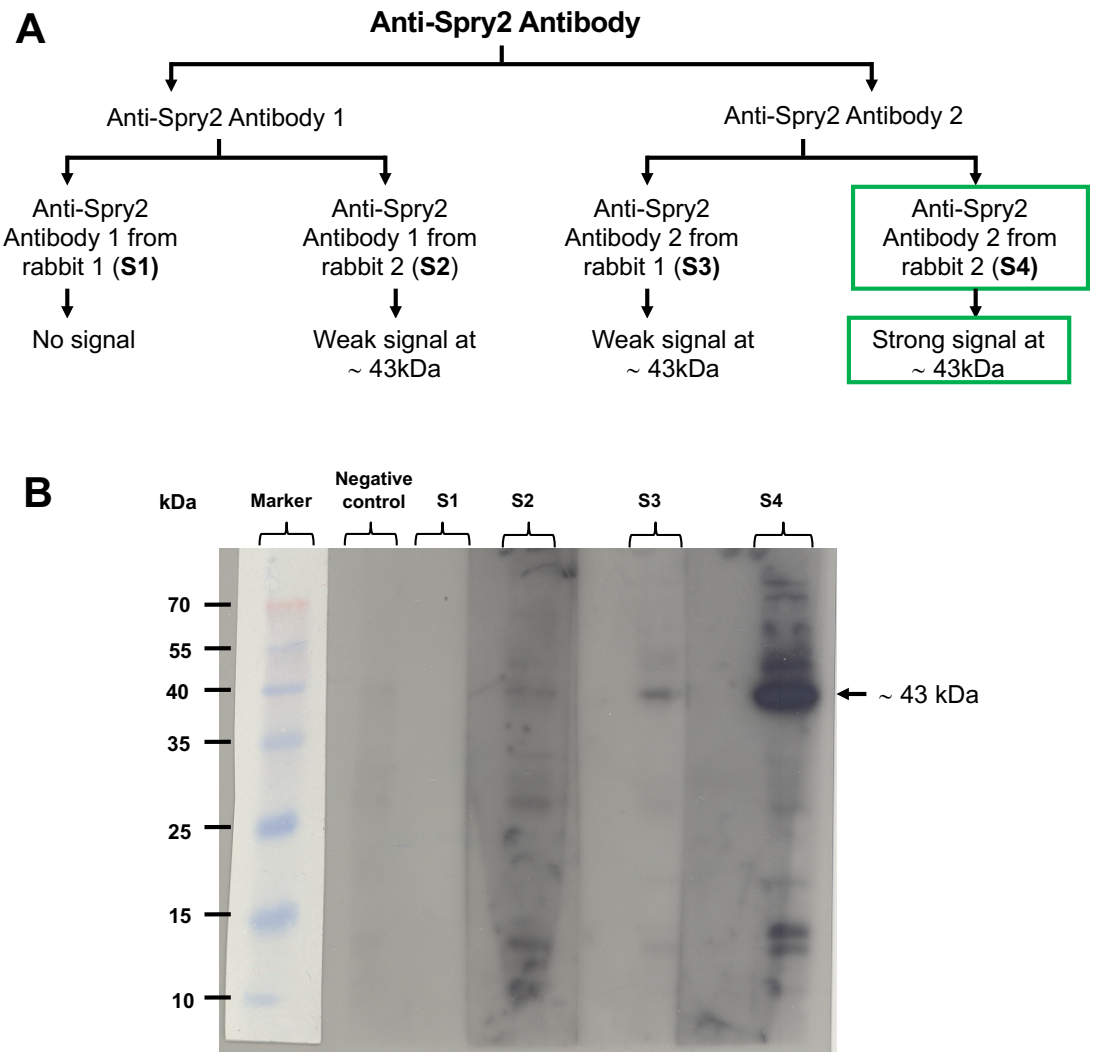

Fig. 23: Western blot analysis of two affinity-purified rabbit anti-Spry2 antibodies. Anti-Spry2 antibody 1 from rabbit 1 (S1), Anti-Spry2 antibody 1 from rabbit 2 (S2), Anti-Spry2 antibody 2 from rabbit 1 (S3) and Anti-Spry2 antibody 2 from rabbit 2 (S4). Samples treated with the secondary antibody only served as a negative control

In a Western blot with whole *Hydra* extract, a strong signal resulted at 43 kDa using the anti-Spry2 antibody 2 from rabbit 2 (S4) (Fig. 23 Lane 6). Anti-Spry2 antibody 1 from rabbit 2 (S2) and Anti-Spry2 antibody 2 from rabbit 1 (S3) also detect a weak signal at about 43.26 kDa. The full-length Spry2 protein sequence consists of 384 amino acids and has a calculated molecular weight of about 43.26 kDa (For antibody sequence refer Fig. S24), which is well in the range of the detected main protein band. Additional weaker bands were seen at approximately 10-15 kDa, 55 kDa, and 70-100 kDa might be unrelated proteins or fragments of Spry (Fig. 23). At present, we cannot distinguish whether Spry2 is fragmented or carries post-translational modifications or whether the antibody recognizes other proteins with similar epitopes as well. The negative control incubated with the

second antibody shows no bands. Therefore, non-specific binding of the secondary antibody excluded.

2.4.3.2.3. The Anti-Spry2 antibody detected Spry2 protein at the bud base

Expression of Spry2 protein was reproducibly upregulated at the bud base (Fig. 21). Observing the bud growth phases i.e. the ten bud stages (Otto and Campbell 1977), the earliest sign of Spry2 protein expression was detected in in stage 6 (Fig. 21 M1 and M2). Spry2 protein was detectable in an increasing number of cells from stage 6 onwards until all cells at the bud base expressed it in stage 8 (Fig. 21 M1 - P1, M2 – P2). From stage 8 onwards, protein expression extended in a ring form around the bud base (Fig. 21 P1 – T1 and P2 – T2). A ring of Spry2 protein expressing cells remain at the bud foot even after detachment of bud (Fig. 21 T1 and close-up in T2). In Immunohistochemistry of single cell preparations, Spry2 protein was detected in endodermal epithelial cells (Fig. 21 W and X).

2.4.3.3. Differential expression in the parent's foot

Spry2 mRNA and protein differentially express in the foot (Fig. 24). For example, in (Fig. 24 A, F, and K) Spry2 expressed in the ring form at the basal disc. The expression ring surrounding basal disk was observed (Fig. 24 B, G, L, Q and V) along with the stained nerve net (Fig. 24 V). A bunch of Spry2 positive cells were detected at the basal disc (Fig. 24 C, H, M, R, and W). Moreover, Spry2 positive cells were also expressed in the scattered (Fig. 24 D, I, N, S, and X) and patchy form in the basal disc (Fig. 24 E, J, O, T, and Y). Localization of Spry2 protein expression in the cell membrane and cytosol (Fig. 24 U, V, W, X, and Y) indicates cellular staining of Spry2.

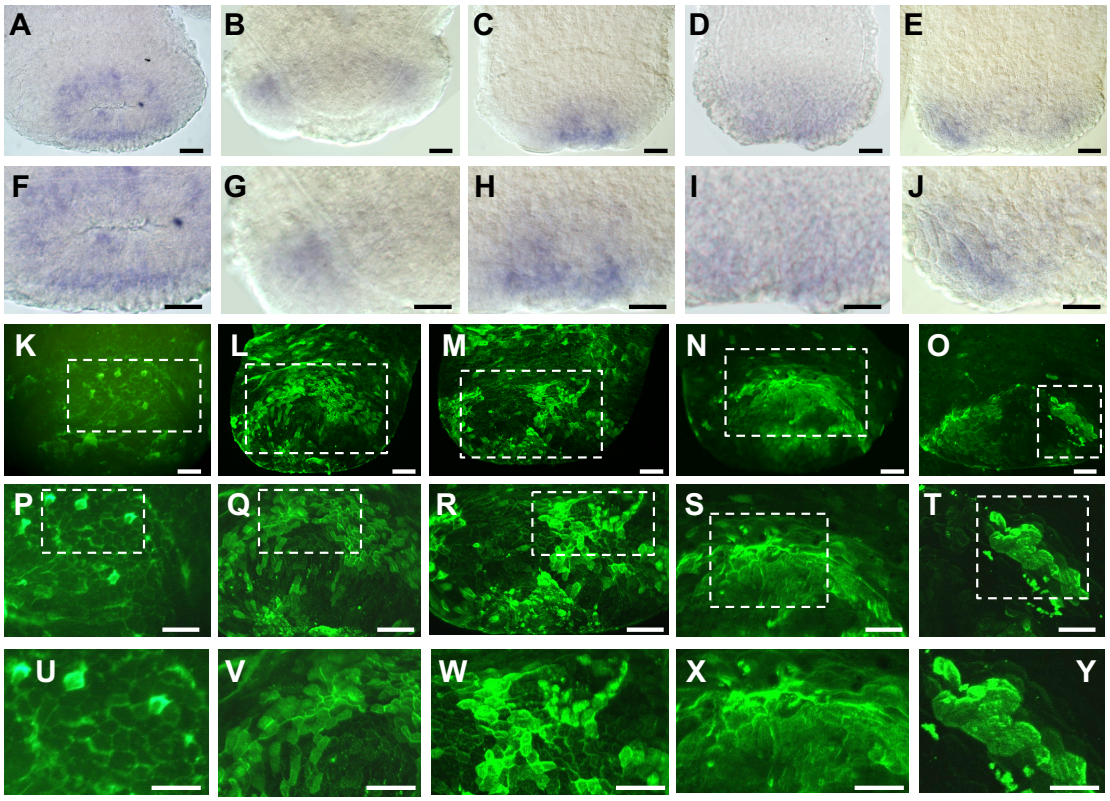

Fig. 24: Differential transcription and expression of Spry2 in the foot. (A-J) mRNA expression of *spry2.* (K-Y) Spry2 protein expression in the foot. (F-J), (P-T) and (U-Y) are close up of (A-E), (K-O) and (P-T) respectively. Scale bar is of 100 µm.

2.4.3.4. *spry2* co-express with *FGFRa (kringelchen)*

To find out whether *spry2* and *FGFRa* (*kringelchen)* are expressed in the same or in different cells, double *in situ* hybridizations were performed using different combinations of Dig-labelled antisense RNA probes such as *spry2* + *FGFRa (kringelchen), spry2* + *MMPA3, spry2* + *MK55, FGFRa (kringelchen)* + *MK55.*

Expression of *spry2* and *FGFRa* (*kringelchen)* (Fig. 25 A-C) are denoted by blue and red color respectively. Transcripts of both genes are detected closely together resulting in a zone of purple color instead of distinct zones of red and blue. This observation suggests the co-expression of *spry2* and *FGFRa (kringelchen)* in the same cells.

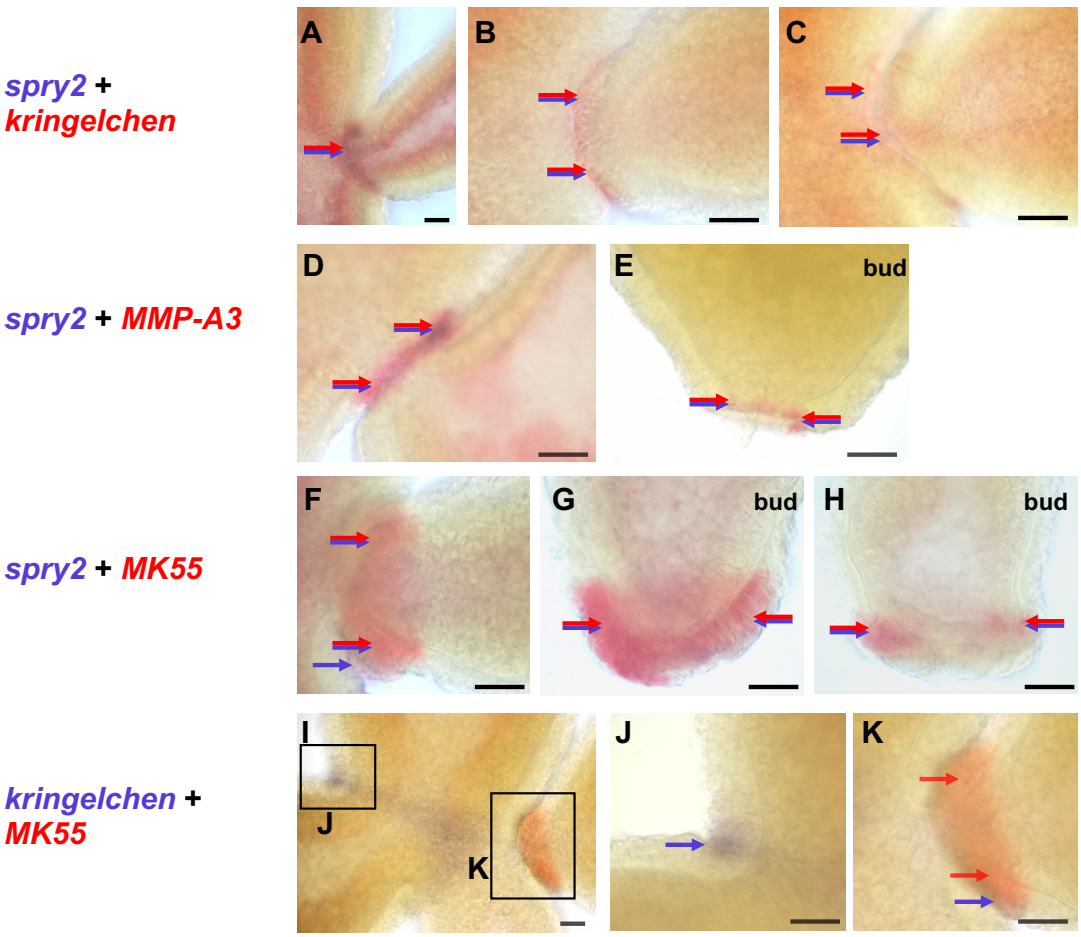

Fig. 25: Double whole-mount *in situ* hybridization for combinations of *spry2*, *FGFRa (kringelchen), MK55* and *MMP-A3* in late budding stages. Double *in situ* hybridizations at bud stages 8-10; (A-C) *spry2* (blue) and *FGFRa (kringelchen)* (red); (D-E) *spry2* (blue) and *MMP-A3* (red); (F-H) *spry2* (blue) and *MK55* (red); (I-K) *FGFRa (kringelchen)* (blue) and *MK55* (red). Staining reactions were performed using either NBT/BCIP (Roche, blue signals) or FastRed (Sigma, red signals). Red and blue color arrow denote red and blue signal respectively. Localization of both arrows close-by indicated co-expression.

In a second experiment, the possible co-expression *of MMP-A3* and *spry2* was studied, as the mRNA for the matrix metalloprotease *MMP-A3* and *FGFRa (kringelchen)* are expressed in the same cells (Münder et al. 2010; Bottger and Hassel 2012). In (Fig. 25 D-E) overlapping expression of *spry2* (blue) and *MMP-A3* (red) is disclosed. Both mRNAs are expressed in the same cells resulting, again, in a purple colored zone at the bud base. Fig. 25 E is a bud, which had been ripped off the parent incidentally prior to its natural detachment and showing the purple ring of *spry2* and *MMP-A3* mRNA colocalization.

Furthermore, expression of a foot differentiation marker gene *MK55* (red) (Hotz 2006), was examined against *spry2* (blue). Transcripts of *spry2* were found in the basal disc (Fig. 24 A-E). In Fig. 25 F, *MK55* does not colocalize with *spry2,* but it is in the lowest possible position of the basal disc. Additionally, *spry2* mRNA is also detectable more peripheral towards the parent. In detached bud, *spry2* is co-expressed with *MK55* (Fig. 25 G and H). Compared to Fig. 21 J2, it becomes clear that in contrast to *MK55, spry2* has an additional expression domain in the parent, in which the mRNA persists for a short time in cells forming a ring around the detachment site. Co-expression of *spry2* and *MK55* confirmed the expression in mature basal disk cells.

Likewise, expression of *FGFRa (kringelchen)* (blue) was assessed against foot differentiation marker *MK55* (red) (Hotz 2006) (Fig. 25 I-K). As cited in (Sudhop et al. 2004) *FGFRa (kringelchen)* is express in the bud foot and persists at the bud detachment site in parental tissue for a short time after bud detachment. In Fig. 25 I, J and K, *FGFRa (kringelchen)* is detected in the base of a stage 6 bud (blue color) (Fig. 25 I, J) as well as at the foot in bud stage 8-9 (Fig. 25 I, K). Expression pattern indicates *FGFRa (kringelchen),* and *MK55* are not in the same cells (Fig. 25 I, K).

Two-color *in situ* hybridization experiments strongly suggest that *spry2* is coexpressed with *FGFRa (kringelchen)* (Fig. 25 A-C). Thus, the precondition for targeting of Spry2 by signaling through the FGFRa (kringelchen) and mutual modulation within one intracellular pathway is given.

2.4.3.5. Expression of *spry2* in the regenerates

Hydra undergoes morphogenesis to form and maintain structures in normal (budding) polyps, and additionally has a high potential to regenerate missing structures. Transcription of the gene of interest during morphogenesis can as well be analyzed using regeneration experiments. Fig. 21 and 24 show that *spry2* is upregulated in the foot, so our questions were when *spry2* is upregulated during foot formation and whether it might also be upregulated during head formation.

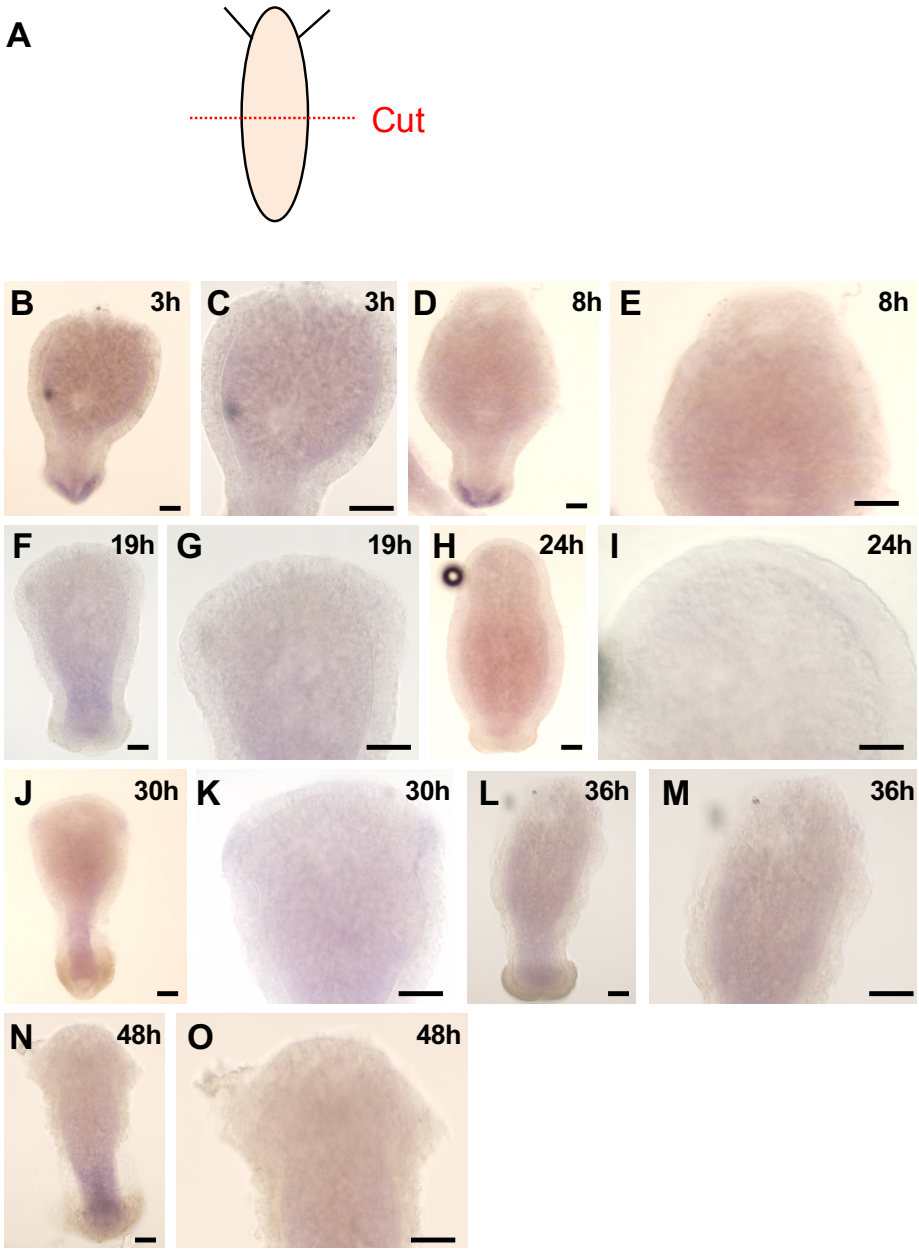

Fig. 26: Time course of *spry2* expression during regeneration of head structures between 3 and 48 hrs. Regeneration time points after sectioning *Hydra* are given in the upper right corner. Scale bar = 100 µm.

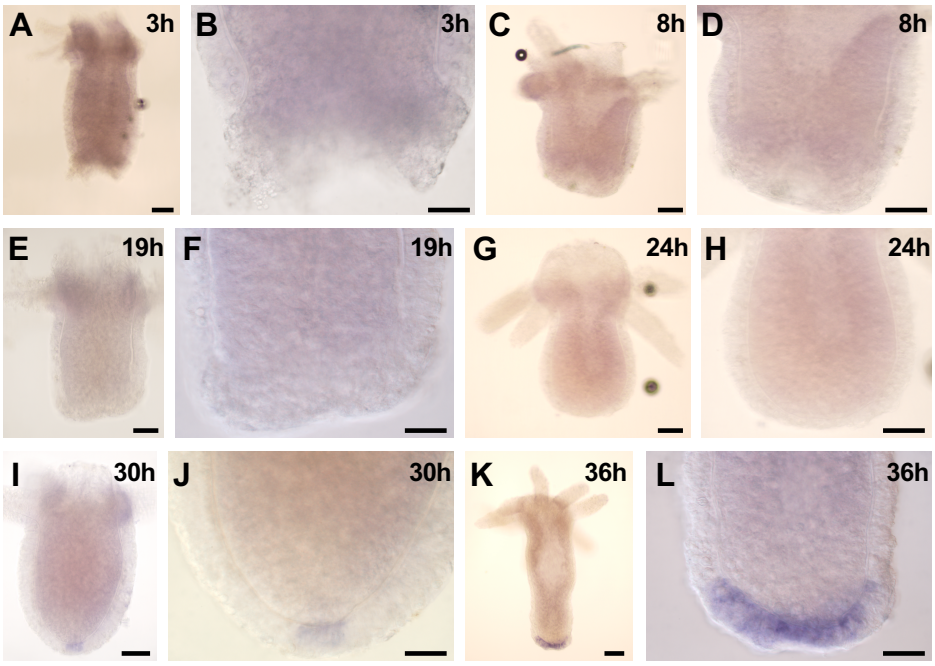

Fig. 27: *spry2* expression in foot regenerating structures between 3 and 36hrs. Regeneration time points after sectioning *Hydra* are mentioned in the upper right corner. Scale bar = 100 µm.

Regeneration was induced by bisection in the middle of the body column and *spry2* expression analyzed at different time points after sectioning. *spry2* transcription was neither induced at regeneration sites early during wound closure nor late in head regenerates (Fig. 26 B-O). The normal *spry2* expression in the basal disc of the tissue fragment was found unchanged throughout head regeneration (Fig. 26 B, D, F, J, L and N).

In contrast, in foot regenerating fragments, the first *spry2* transcripts became detectable from 30 hrs onwards in a small, basal most group of ectodermal cells of the regenerating foot and thereafter extended laterally (Fig. 27 I, J, K, and L). *Hydra* mature foot comprised of a peduncle with a flat bottom basal disc at its end (Martinez and Bridge 2012). A basal disk of *Hydra* is simple constant size disk shaped, with two layers of epithelial cells, i.e. the endodermal epithelium and the ectodermal epithelium (Amimoto et al. 2006). From 30hrs of regeneration and preceding structure formation by 6 hrs *spry2* expressed in the regenerating foot ectoderm, indicates the possible role of *spry2* in foot differentiation (Fig. 27 I, J, K, and L).

2.4.4. The level of *spry2* expression seems to depend on FGFR signaling

The expression pattern of *spry2* in budding and regenerating *Hydra* suggested a possible function in bud detachment or foot differentiation. In vivo inhibition studies were performed to investigate, whether Spry2 is targeted/modulated by FGFR or modulates FGFR activity within the FGFR signaling pathway? Specific inhibitors against two different downstream elements within the FGFR pathway exist (Fig. 28). SU5402 was used to specifically inhibit FGFR kinase activity (Mohammadi et al. 1997). The second target for inhibition was MEK, which fails to phosphorylate ERK when inhibited by U0126 (Duncia et al. 1998; Favata et al. 1998). Inhibition experiments with SU5402 and U0126 were performed under my supervision by Jens Westermann during his bachelor project.

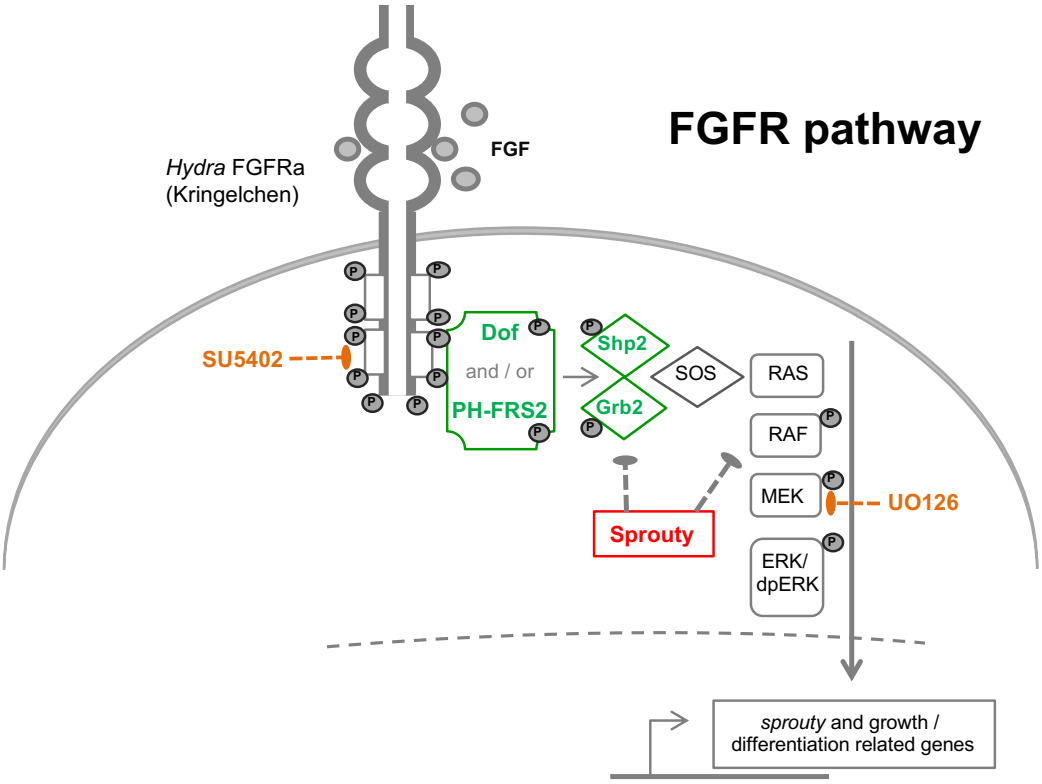

Fig. 28: Functional study of Spry2. Schematic view of FGFR pathway. FGFR pathway inhibitor molecules SU5402, U0126 and their target sites are indicated in orange color.

2.4.4.1. FGFR inhibitor (SU5402) reduced *spry2* expression

SU5402 is an FGF receptor inhibitor, which blocks tyrosine kinase activity of all four vertebrate FGFRs by interacting with the catalytic domain and thereby blocks

FGF signaling (Mohammadi et al. 1997) (Fig. 28). To investigate, whether a direct regulation of Spry2 expression by FGFR signaling occurs, a time-course analysis was performed by incubating budding *Hydra* polyps with 10µM SU5402 as described previously (Sudhop et al. 2004). Animals incubated in DMSO/ATP solution (control 1 Fig. 29 D and H) or in *Hydra* medium (control 2 Fig. 29 E and I) were used as a control. Expression of *FGFRa (kringelchen)* in SU5402 treated animals (Fig. 29 J, K, N, and O) and control animals (Fig. 29 L and M) was evaluated to confirm, that inhibition of FGFR in *Hydra vulgaris* AEP affects FGFRa expression as shown by (Sudhop et al. 2004) for *Hydra vulgaris, Zürich*. The switch to *Hydra vulgaris* AEP had to be done because this is the only *Hydra* strain, which successfully undergoes transgenesis (Wittlieb et al. 2006) and in the long term will be used for functional studies using transgenic polyps.

After inhibitor treatment, buds developed normal but failed to detach. Bud of stage 2-3 was undergoing treatment exhibited Y-shaped animals (Fig. 29 B, C, J, and K). Inhibition of FGF receptor yielded reduced *spry2* expression (Fig. 29 B, C, F, and G). While normal animals together with DMSO treated animals had normal expression of *spry2* (Fig. 29 D, E, H, and I). In normal animals *spry2* express in the foot (Fig. 24). As first described by (Sudhop et al. 2004; Münder et al. 2010), in SU5402 treated animals, *FGFRa (kringelchen)* expression was distorted (Fig. 29 K and O) or missing (Fig. 29 J and N), and additionally bud detachment was inhibited (Fig. 29 J, K, N, and O). The data show that *Hydra vulgaris* AEP reacts to FGFR inhibition just like *Hydra vulgaris, Zürich.*

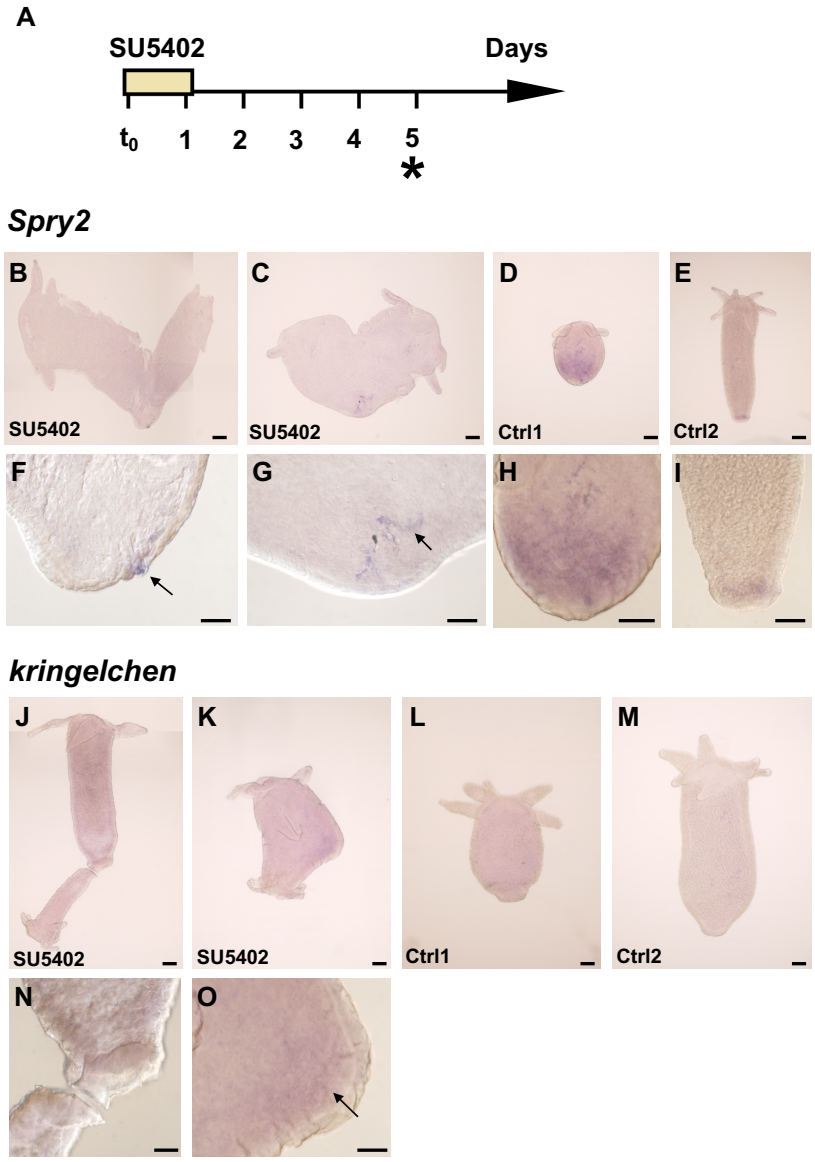

Fig. 29: *spry2* and *FGFRa (kringelchen)* expression after treatment with FGFR inhibitor SU5402. (A) SU5402 treatment scheme. Buds at stage 3 were treated for 24 hours with SU5402 and subjected to WMISH five days later (asterisk). (B-I) *spry2* expression in SU5402 treated animals, (J-O) *FGFRa (kringelchen)* expression in SU5402 treated animals. Animals incubated in DMSO/ATP solution was used as a control 1 and animals in *Hydra* medium was used as a control 2. Scale bar = 100 µm.

Another control experiment was performed to reconfirm the *spry2* expression in SU5402 treated animals (Fig. 30 A-M). According to (Münder et al. 2010), the metalloprotease *MMP-A3* is co-expressed with *FGFRa (kringelchen)*. Therefore, WMISH using probe against *MMP-A3* was additionally carried out to investigate coexpression with *FGFRa (kringelchen)* and ensure comparability of the experiments.

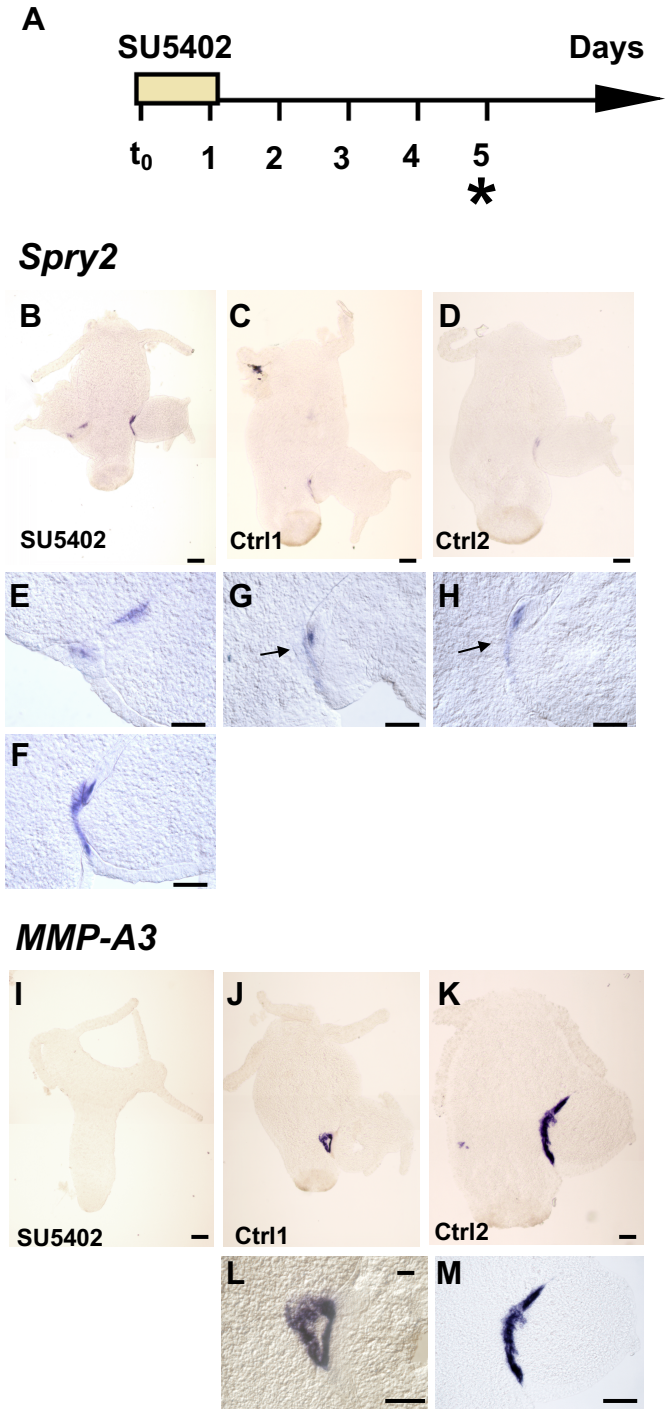

Fig. 30: *spry 2* and *MMP-A3* expression after treatment with inhibitor SU5402. (A) SU5402 treatment scheme. Buds at stage 3 were treated for 24 hours with SU5402 and subjected to WMISH five days later (asterisk). Animals incubated in DMSO/ATP solution was used as a control 1 and animals in *Hydra* medium was used as a control 2. (B-H) *spry2* and (I-M) *MMP-A3* expression in SU5402 treated and control animals. Scale bar = 100 µm.

Analysis of *spry2* expression in SU5402 treated polyps carrying a stage 3 bud (Fig. 30 B and D) showed reduced expression of the gene. No such effect was observed in treated late buds (Fig. 30 B and F). In DMSO-treated and untreated animals, *spry2* was found expressed at the normal level in ring form at the bud base (Fig. 30 C, D, G, and H). Expression of *MMP-A3* was inhibited in SU5402 treated animals (Fig. 30 I) while expressed normally in control animals (Fig. 30 J, K, L, and M).

2.4.4.2. MEK inhibitor (U0126) did not influence *spry2* expression

U0126 specifically inhibits MEK1 and MEK2 (Duncia et al. 1998; Favata et al. 1998). In the *Hydra* FGFR pathway, U0126 inhibits ERK phosphorylation and delays bud detachment (Hasse et al. 2014). Since Spry expression can be induced through RTK signaling mediated by FRS2 or Dof and subsequent activation of the RAS-ERK MAPK pathway (Masoumi-Moghaddam et al. 2014), *spry2* gene expression was investigated following MEK inhibition.

Budding *Hydra* were treated with U0126 as mentioned in (Hasse et al. 2014). As a control, animals incubated in the DMSO/ATP solution (control 1 Fig. 31 D and H) or in *Hydra* medium (control 2 Fig. 31 E and I) were used. Inhibition delayed bud detachment as inhibitor treated buds are just in stage 8 (Fig. 31 B and C) while untreated buds are at stage 10 (Fig. 31 D and E). The inhibition of detachment did not influence bud morphology and had no detectable effect on *spry2* expression (Fig. 31 B, C, F, and G). The position of the polyp's bud in Fig. 31 B at the foot of the parent is unusual because it remained attached to the parent, but shifted towards the parent foot, although a stage 9 bud were treated. Typically, a non-detaching bud is shifted towards the basal disc of the parent polyp within 3-4 weeks just following the normal mass tissue movement. Therefore, this fast shift might be an effect of the MEK inhibition. Since this effect was observed only in a single animal, it was not followed further.

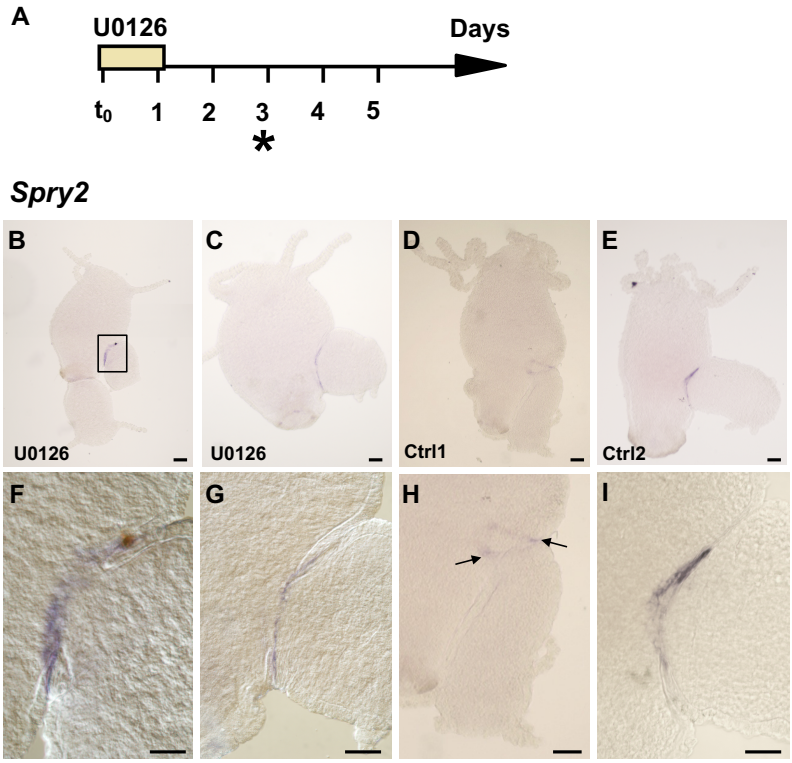

Fig. 31: *spry2* expression after treatment with MEK inhibitor U0126. (A) U0126 treatment scheme. Buds at stage 8-9 were treated with U0126 for 24 hours and subjected to WMISH five days later (asterisk). Animals incubated in DMSO/ATP solution was used as a control 1 and animals in *Hydra* medium was used as a control 2. (B-I) *spry2* expression in U0126 treated and control animals. Scale bar = 100 µm.

3. Discussion

3.1. Docking proteins

3.1.1. Receptor tyrosine kinases

The glimpse of receptor tyrosine kinases (RTK) in the common ancestor of choanoflagellates and Metazoa (Pincus et al., 2008) was a considerable achievement in evolution studies. The novel combination of domains to build a membrane-anchored receptor tyrosine kinase protein, which receives extracellular ligands and directly transmits them via the intracellular tyrosine kinase activity described an exceptional model of receiving and transducing signals into cells. Even if RTKs are predicted in single celled and multicellular Choanoflagellata, they are not directly related to metazoan RTKs and ligands are not yet known (Pincus et al., 2008). In fact, strong evidence has been given recently for fibroblast growth factors (FGFs) and their receptors (FGFR) in metazoa (Bertrand et al., 2014; Rebscher et al., 2009). Although in the sponge (*Oscarella*) and in the parazoan *Trichoplax*, FGFRs were predicted, FGF-encoding sequences could not be identified and occur in Cnidaria for the first time (Bertrand et al., 2014; Lange et al., 2014; Rentzsch et al., 2008).

Specific docking proteins are essential for FGF receptor tyrosine kinase to signal through PI3 kinase and RAS/MAPK pathways (Guy et al. 2002; Thisse and Thisse 2005). FRS2 in vertebrates and Dof in *Drosophila,* are two completely unrelated docking proteins (Thisse and Thisse 2005; Muha and Muller 2013). Since there is no data available about which docking proteins act in other invertebrates, the question emerged, when docking proteins originated and which one exists in *Hydra* to trace the evolution of FGFR signaling events. The genes encoding both potential FGFR docking proteins were identified in *Hydra*, their evolution, and transcription patterns will be discussed in the following.

3.1.2. Vertebrate and invertebrate specific docking proteins in Cnidaria

In early-diverged Eumetazoa, FGFR tyrosine kinases play an essential role during morphogenesis (Sudhop et al. 2004; Rentzsch et al. 2008; Hasse et al. 2014), but it is still unknown how the signals are conveyed into the cells. The first evidence of invertebrate-specific docking protein Dof in Cnidaria, has been reported

here. Despite the identification of highly conserved domains in Dof orthologues of *Hydra* and Bilateria, sequences encoding a DBB domain with convincing similarity could not be identified in gene databases of the placozoan *Trichoplax* and of choanoflagellates. This finding is surprising because receptor tyrosine kinases evolved in choanoflagellates and Metazoa (King et al. 2008; Pincus et al. 2008; Fairclough et al. 2013). Although choanoflagellate RTK's differ from metazoan RTK one might expect them to use docking proteins, which are required in all metazoans to enable signal transduction through selected downstream pathways (Lemmon and Schlessinger 2010; Bertrand et al. 2014). Results indicates FRS2 docking proteins required in taxa thought to have derived from the main line of animals before the Cambrian, namely in *Trichoplax* (Parazoa), in *Amphimedon* (Porifera) and in the sister group of animals, the choanoflagellates, which indicates an early complete toolkit for RTK signaling. Our investigation shows that both potential FGFR docking proteins found in Bilateria, namely Dof in fly and FRS2 in vertebrates, exist in Cnidaria.

3.1.3. Dof, a putative eumetazoan invention

Cnidaria docking proteins Dof in *Acropora* and *Hydra* possesses unique domain architecture (DBB domain + ankyrin repeats + binding sites for Grb2, Shp2, PI3K) (Fig. 6). The presence of both Grb2 and Shp2 proteins in the *Hydra* database indicates that the toolkit used by fly FGFRs is also available for cnidarian FGFRs. Counting on all the domains that are present and well aligned, *Acropora* and *Hydra* Dofs have the potential to act as a docking protein.

Dof could neither be found in Porifera nor in Placozoa, which suggests that Dof is either an innovation in the last common ancestor of Cnidaria and Bilateria, or it has been lost in the earlier derived taxa. The first possibility correlates to clearly identifiable FGFRs and their described functions in Cnidaria (Sudhop et al. 2004; Rentzsch et al. 2008; Hasse et al. 2014). A drawback is that Dof is missing in anthozoan *Nematostella*, while the anthozoan coral *Acropora* possesses Dof. Our data also show that BCAP and BANK proteins are very likely vertebrate innovations. vertebrates use the Dof domain proteins BCAP and BANK for immunoregulation (Battersby et al. 2003). The phylogenetic tree indicates that in fact, Cnidarian Dof ancestral one but *Acropora* seems derived for some reason.

Only in insects, Dof has been identified as a functional and indispensable FGFR docking protein (Vincent et al. 1998; Csiszar et al. 2010). In the future, it would be interesting to investigate which taxa, in fact, use Dof to couple FGFR to intracellular signaling cascades and whether this function correlates with this tree topology. In summary, our data suggest the first appearance of Dof in the last common ancestor of Cnidaria.

3.1.4. A novel family of potential invertebrate docking proteins, PH-FRS2

It is mysterious, why insects use Dof and vertebrates use FRS2, exclusively, to dock activated FGFR to downstream pathways. Both vertebrates and invertebrates possess Dof as well as FRS2 (Lo et al. 2010) (Fig. 6 and 11). Analysis of the evolution of FRS2 turned out quite interesting because it revealed the mutually exclusive presence of either FRS2 – or PH-FRS2 in animal phyla in a very puzzling pattern. Both belong to the superfamily of membrane-linked-docking proteins (MLDP) which also comprise insulin receptor binding proteins (IRS) and Dok (Downstream of tyrosine kinase) proteins. Both these groups in vertebrates bind insulin receptor or EGFR, SHIP or other Dok proteins by their PTB domain and they activate Grb2, Shp2 or RAS-GAP (Gotoh 2008). Our study now revealed a new member of this family in Placozoa, Cnidaria, Annelida and Mollusca, which we named PH-FRS2. Phylogenetic tree (Fig. 14 and 15) topology and placement of the basal taxa (Choanoflagellata, Porifera, Placozoa, Cnidaria) indicates that FRS2 originated in the last common ancestor of choanoflagellates and Metazoa. PH-FRS2 occurred in Metazoa, while the two groups of Dok proteins (Dok 1-3 and Dok 4-6) likely originated in the last common ancestor of Cnidaria and Bilateria and IRS proteins in the last common ancestor of Bilateria. *Nematostella* proteins are always placed basally (within the PH-FRS2, Dok1-3, and Dok 4-6 groups), while the *Hydra* PH-FRS2 proteins appear derived, consistent with the evolution of FGFR intron patterns, conserved genome (Rebscher et al. 2009). Dok4 and Dok5 are also known as IRS5 and IRS6 and bind insulin receptor by unknown residues was not corresponding to the ones identified in IRS1 (Cai et al. 2003). The question whether *Hydra* IRS-like potentially binds *Hydra* insulin-receptor (Steele et al., 1996) could not be solved by comparison of IR binding sites in IRS1 and *Hydra* IRS-like (Fig. S11). *Hydra* IRS-like lacks the IRS1 binding residues, but like Dok4/IRS5 and Dok5/IRS6

might bind IR using different sites. Further studies are needed to determine, whether an explanation can be found for mutually exclusive occurrence of FRS2 and PH-FRS2 proteins. It is also not possible at present to discuss a function of PH-FRS2 in Cnidaria. Data from Cnidaria other than *Hydra* are not available. Gene expression in *Hydra* at the tentacle bases of buds and parent animals is very weak, and conditions increasing its transcription could not be found yet.

3.2. Presence of Grb2 and Shp2 in *Hydra*

Our study revealed the essential docking proteins of the *Hydra* FGFR pathway, the subsequent goal was to identify further downstream elements like Grb2 and Shp2 needed for transmission of signal further to RAS/ MAPK pathway. Sequence analysis discovered one Grb2 protein with signature domains such as two SH3 and one SH2 domain known from Grb2 proteins in other animals (Clark et al. 1992; Stern et al. 1993; Downward 1994). Grb2 proteins containing SH2 and SH3 domains link the receptor tyrosine kinase to RAS/ MAPK signaling (Lowenstein et al. 1992; Kouhara et al. 1997). Literature and our finding anticipate involvement of Grb2 in *Hydra* FGFR pathway. However, in future, it needs to be examined experimentally.

Two Shp2 (SH2 domain-containing tyrosine phosphatase 2) like proteins were found in *Hydra* - one protein is similar to mammalian PTPN11 protein (Freeman et al. 1992), and another one is like invertebrate Csw protein (Perkins et al. 1992). Compared to known PTPN11 and Csw, both *Hydra* Shp2 proteins contain specific PTP and SH2 domains, with the exception of one SH2 domain missing in *Hydra* Csw-like. SH2 domains mediate Shp2 interactions with RTKs and Grb2 (Noguchi et al. 1994; Cunnick et al. 2001; Cunnick et al. 2002; Neel et al. 2003; Tajan et al. 2015) and PTP directs dephosphorylation (Neel et al. 2003; Tajan et al. 2015). Shp2 orthologs in *Drosophila* (Csw), *Caenorhabditis elegans* (Ptp-2) and vertebrates (PTPN11) have variable numbers of potential tyrosyl phosphorylation sites (Y) and/or prolyl-rich sequences in their C-terminal tails. The *Drosophila* Csw has a cysteine and serine-rich insert within its PTP domain (Neel et al. 2003). In summary, *Hydra* possess both vertebrate and invertebrate specific Shp2 orthologs. Its puzzling why *Hydra* carry both Shp2 orthologs while *Nema-*

tostella carries only one ortholog i.e. PTPN11 (Fig. S13). In future study is required to understand whether both Shp2 orthologs play active role in *Hydra* FGFR pathway.

3.3. Negative regulator - Spry

3.3.1. Cnidarian Spry is the most ancestral one known to date

All four *Hydra* Spry proteins carry signature domains such as Cbl TKB binding motif, a tyrosine phosphorylation site, spry domain and the Raf1-binding domain as described for bilaterian Spry proteins (Fig. 19) (Hacohen et al. 1998; de Maximy et al. 1999; Mason et al. 2006; Guy et al. 2009). Phylogenetic analysis placed *Hydra* and *Nematostella* Spry at the base. In phylum Cnidaria class Anthozoa is more ancient, and Hydrozoa class is more derived (Steele et al. 2011). We found Spry in both, the Anthozoa and Hydrozoa classes of Cnidaria. Although we were able to identify cDNAs encoding Sprouties with highly conserved RBD and Spry domains in *Hydra*, no such evidence was found in the genome of the placozoan Trichoplax or the choanoflagellate genomes. It is somewhat surprising, given the high conservation and essential role of growth factor receptor tyrosine kinases in animals and the fact that RTKs are encoded in the genomes of both taxa (Bertrand et al. 2014). RTK are thought to have evolved in choanoflagellates, the sister group to animals, but they lack recognizable members of the RTK families present in Cnidaria and Bilateria consistently (King et al. 2008; Pincus et al. 2008; Fairclough et al. 2013). Therefore, those early RTKs might not use comparable negative feedback mechanisms.

3.3.2. A putative link between FGFR signaling and Spry

Regulation of Spry function, stability and localization depend on many interacting and associated proteins such as Grb2, Shp2, Cbl-b, FRS2, Raf1, PTP1B, etc (Mason et al. 2006; Cabrita and Christofori 2008; Edwin et al. 2009). Spry function is difficult to analyze because several kinases (Src, Mnk1) and phosphatases (PP2A) are involved in complex regulation loops (Mason et al. 2006; Cabrita and Christofori 2008; Edwin et al. 2009; Guy et al. 2009). According to several studies, the *spry2* transcript expression is reduced after treatment with the FGFR inhibitor SU5402 (Komisarczuk et al. 2008; Moura et al. 2011). Similarly, *Hydra spry2* mRNA expression was also reduced upon treatment with SU5402. The effect is

specific because *Hydra* possesses seven out eight identical amino acids described essential for SU5402-FGFR binding (Sudhop et al. 2004). Results suggest the position of Spry2 at downstream of *Hydra* FGFR, but yet its function in *Hydra* FGFR pathway is unknown. However, we cannot exclude the possibility that other signaling pathways may be inhibited in these experimental conditions, also contributing for *Hydra* morphology after inhibitor treatment.

3.4. A toolkit for FGFR signaling: Dof, FRS2, Grb2, Shp2 and Spry2

The expression analysis of *FGFRa (kringelchen)* and *dof* transcripts failed to show overlapping expression domains (Fig. 32). FGFR appears from the earliest stage onwards at the bud tip and later resides at the bud base, exclusively (Sudhop et al. 2004; Bottger and Hassel 2012). *FGFRa* is additionally expressed at a low level throughout the body column (Sudhop et al. 2004). Localization of *dof* transcripts outside the strong *FGFRa* expression zones, at first glance, excludes interaction of Dof and FGFR (Fig.32). However, since *FGFRs* are expressed at a low level throughout the body column, protein-protein interactions might be possible in regions of low FGFR expression, provided, *Hydra* FGFRs require a docking protein at all.

3.4.1. Dof, an interesting candidate for attraction and local differentiation signals in *Hydra*

FGFRa (Kringelchen) might not just play a role in bud detachment (Sudhop et al. 2004), but could also function in the upper body region, where a particularly interesting function might be a role in cell attraction and cell differentiation (Lange et al. 2014). In *Hydra*, the stationary large interstitial stem cells e.g. neuronal and nematocyte (stinging cells) (Boehm and Bosch 2012) of the mid-body region become committed to fates. Interstitial stem cells migrate as differentiating precursor cells towards the head, where they integrate into proper places, e.g. preferably into the battery cells of tentacles (nematoblast cells) (David 2012; Hobmayer et al. 2012). The molecules which attract interstitial cells towards the head and control their differentiation are unknown. As shown in vertebrate and fly, FGFs are very interesting candidates as chemoattractants and differentiation signals. A model (Bae et al. 2012) suggests that high concentrations drive differentiation and low FGF concentrations attract cells.

Hydra FGFf, an FGF8-homolog, is expressed at all *Hydra* termini and boundaries through which cells have to migrate including the tentacle bases (Lange et al. 2014). In FGFR pathway, Dof may dock FGF/FGFR tetramers to specific intracellular pathways allowing terminal differentiation of nematocytes and possibly even of the epithelial battery cells. This cell population is differentiated from epithelial cells of the body column, they enlarge and take up differentiated nematocytes in a well-arranged pattern to be used to kill or bind prey. Thus *Hydra dof* with its transcript localization close to the tentacle bases, is an interesting candidate for further investigation of a (local) role of FGFR signaling in a non bilaterian animal.

3.4.2. FGFR and Spry expression follow each other suggesting a potential functional relationship

Spry was originally identified as an antagonist of FGF signaling, which patterns apical branching in *Drosophila* tracheae (Hacohen et al. 1998). Furthermore, *Drosophila* having mutated *spry,* produced excessive FGF signaling induced ectopic airway branches (Kim and Bar-Sagi 2004). Hence Spry, as an intracellular negative regulator of FGFR signaling, attenuates the FGFR signal by interfering with the activity of several intracellular targets (Guy et al. 2003; Kim and Bar-Sagi 2004; Guy et al. 2009). So, one can expect Spry as a downstream element and target of FGFR signaling. Previous studies revealed, the *spry2* transcript expression is reduced or even abolished after treatment with the FGFR inhibitor SU5402 (Komisarczuk et al. 2008; Moura et al. 2011). In summary, subsequent expression of *spry2* behind the *FGFRa* and reduction of *spry2* expression in FGFR inhibitor-treated animals supports the literature; suggesting Spry2 as a downstream element of FGFR signaling (Fig. 32) and a possible potential role of Spry2 in bud detachment.

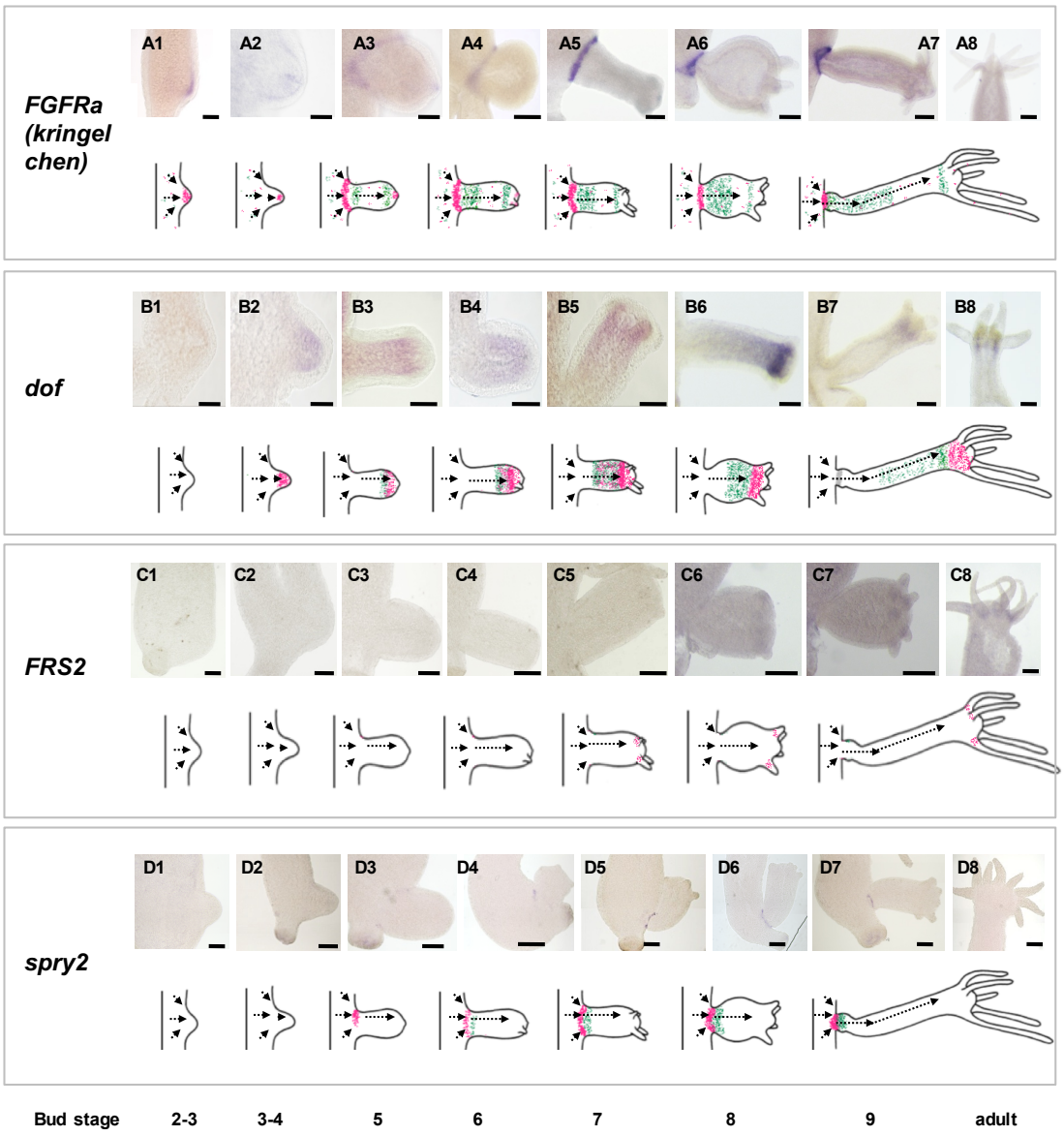

Fig 32: A model for putative protein localization with respect to transcript expression profile of *Hydra FGFRa, dof*, *FRS2* and *spry2*. The mRNA expression indicated with pink color and putative protein expression denoted with green color. The direction of the tissue movement indicated with arrows.

Schematic representation of putative protein localization drawn concerning expression domains of *FGFRa (kringelchen), dof* and *spry2* at different bud stages (Otto and Campbell 1977) (Fig. 32). The mRNA expression and anticipated tissue movements (e.g. into the bud body indicated by arrows), were considered to develop a model for the putative protein localization. In early developing buds, *FGFRa (kringelchen)* co-localizes with *dof* in the head region (from stage 3 to stage 9), while in late buds (from stage 5 to stage 10 and in parent after bud detachment) it co-localizes with *spry2* in the bud base. Unfortunately, it has not yet been possible to localize the native FGFRa (kringelchen) protein. Several

studies in *Drosophila* and vertebrates have reported, expression of Dof and Spry is coincident with the known sites of FGF signaling (Hacohen et al. 1998; Michelson et al. 1998; Vincent et al. 1998; Imam et al. 1999; Minowada et al. 1999; Tefft et al. 1999; Chambers and Mason 2000). Furthermore, previous studies also noted the Dof and Spry as both component and downstream targets of the FGF signaling pathway (Horowitz and Simons 2008, 2009; Muha and Muller 2013). In *Hydra, dof* and *spry2* expression follows to *FGFRa (kringelchen)* expression; this supports the hypothesis that Dof and Spry2 are downstream elements and targets of FGFR signaling.

Moreover, previous studies have reported weak expression of *FGFRa* throughout the bud (Sudhop et al. 2004), which might have made *dof* transcript to express along with. However, (Sudhop et al. 2004) have reported the persistent expression of *FGFRa* at the bud base. Considering the *Hydra* tissue dynamics (), it can be speculated that the bud cells might have carried the FGFRa protein from the bud base encouraging dof expression (Fig. 32). Cells in the immortal *Hydra* consistently proliferate thereby generating a tissue movement towards the apical and basal termini as well as into vegetative buds. Epithelial cells are shifted towards the boundaries of the animal and into outgrowing buds (Aufschnaiter et al. 2011). Thus cells previously at the evaginating bud tip end up in the bud head, and all cells of the bud cross the FGFR-positive region at the bud base. Therefore, the possibility exists that cells having crossed the region between parent and bud retain or even translate the *FGFR* (despite a low level of expression) and only later upregulate transcription (or mRNA stability) of the docking proteins Dof or FRS2 and of the negative regulator Spry (Fig. 32). This model suggests that, Dof and Spry2 are downstream elements of FGFR signaling.

3.5. Two independent FGFR pathways in *Hydra* – outcome and outlook

In previous studies, several elements of a *Hydra* FGFR signaling pathway, FGFRa and FGFRb receptors (Rudolf et al., 2013), FGF ligands (FGFa, FGFe, FGFc, and FGFf) (FGFa, FGFe, FGFc, and FGFf) (Sudhop et al. 2004; Hasse et al. 2014; Lange et al. 2014 and potential downstream elements such as PKC, PI3 Kinase have been identified (Bosch et al. 1995; Hassel 1998; Hassel et al. 1998; Fabila et al. 2002; Arvizu et al. 2006; Manuel et al. 2006; Hasse et al. 2014) (Fig. 5). Moreover, a clear connection of FGFRa to the Ras, MEK and ERK pathway

as well as to a pathway affecting actomyosin dynamics was indicated (Hasse et al. 2014) (Fig.33). My PhD project now identified several potentially essential downstream elements like homologues of the docking proteins Dof and FRS2, the adapter protein Grb2, the tyrosine phopshatase Shp2 and the negative regulator Spry2.

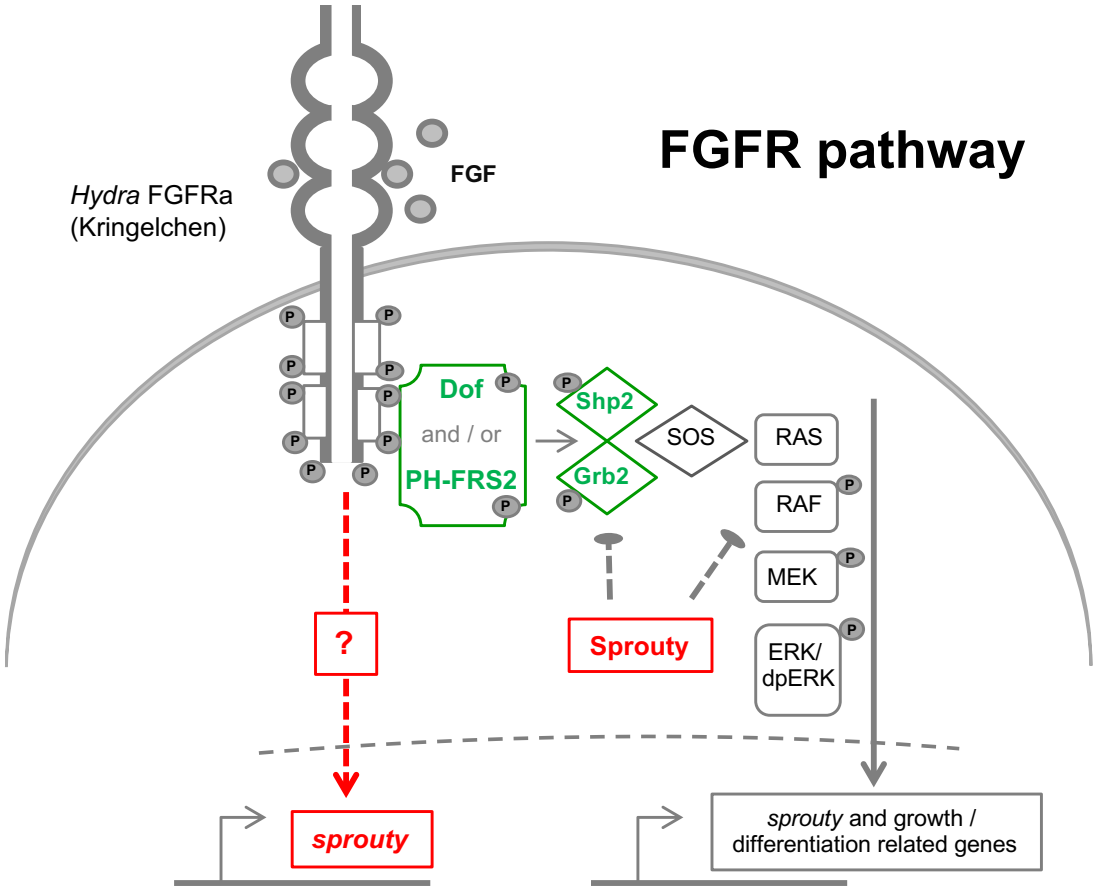

Fig. 33: Downstream elements found in FGFR signaling of *Hydra*

Considering transcript and protein (putative or known) expression of FGFRa, dof, FRS2 and spry2 (Fig. 32), an attempt was made to justify presence of a complex *Hydra* FGFR pathway. Dof (strongly) and FGFRa (weakly) coexpress in the bud head region, indicating that Dof might, like in insects, be a downstream element of FGFR signaling. A region specific involvement of Dof in FGFR dependent cell migration and differentiation is an interesting working hypothesis for further studies. FRS2 expression, in contrast, is too weak at the parent's tentacles bases, to draw any conclusion. The presence of *Dof*, *Grb2* and *Shp2* genes in the *Hydra* genome provides a potential link between FGFRa, docking proteins and the

Ras/MEK/ERK pathway. To test this hypothesis, protein expression level studies should be performed.

Since *Spry2* is upregulated late at the bud base, whereas *Dof* comes up in the bud head region and is constitutively expressed there in all *Hydra* polyps, their involvement in the same pathway is doubted (Fig. 32 and 33). Spry2 and FGFRa, on the other hand, are present in the same cells at the bud base which suggests that FGFRa activates Spry2 as in the insect and vertebrate systems, perhaps even independent of docking proteins (Fig. 33). The involvement of Spry2 as a modulator of FGFR signaling at the bud base needs to be studied carefully in the future.

Understanding the FGFRa protein expression profile will be useful to study *Hydra* FGFR signaling. There are still many open questions such as, whether FGFR signaling is activated in the head region and what its function is, whether *Hydra* FGFRs require docking proteins at all and particularly, how FGFR signaling is modulated in the polyps. Our data suggest the possibility of two FGFR pathways in *Hydra*, one essential for bud detachment and involving FGFRa and Spry2, the other in the (bud) head region involving FGFRa and potentially Dof. Careful investigations using measurement of protein-protein interactions and transgenic polyps are necessary to answer these questions.

4. Material and methods

4.1. Material

4.1.1. Software

Name	URL and Reference
MEGA 5.0	http://mega.software.informer.com/5.0/
MrBayes 3.1.2	http://mrbayes.sourceforge.net/index.php
DOG 1.0	http://dog.biocuckoo.org/down.php
clustalX	http://www.clustal.org/clustal2/
ProbCons version 1.12	http://probcons.stanford.edu/download.html
Jal view version 2.8	http://www.jalview.org/General/General-news/Jalview-28-release-and-the-new-look-wwwjalvieworg
Genedoc	http://genedoc.software.informer.com/
MAFFT version 7	http://mafft.cbrc.jp/alignment/software/
tcoffee	http://tcoffee.crg.cat/
ProtTest version 3.3	(Darriba et al. 2011)
Figtree version 1.4.0	http://tree.bio.ed.ac.uk/software/figtree/
i-Tasser	http://zhanglab.ccmb.med.umich.edu/I-TASSER/
Leica LAS	http://www.leica-microsystems.com/products/microscope-software/software-for-materials-sciences/details/product/leica-application-suite/
Adobe photoshop elements 10	https://helpx.adobe.com/photoshop-elements/kb/photoshop-elements-downloads.html
ImageJ	http://imagej.nih.gov/ij/

4.1.2. Web resources

Name	URL
NCBI	http://www.ncbi.nlm.nih.gov
Hydrazome/ Metazome	http://hydrazome.metazome.net/cgi-bin/gbrowse/hydra
Compagen	http://www.compagen.org
JGI	http://jgi.doe.gov/
NCBI CD search tool	http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi
ExpasyProsite	http://prosite.expasy.org/
Pfam	http://pfam.sanger.ac.uk/

Motif scan	http://myhits.isb-sib.ch/cgi-bin/motif_scan
PhosphoMotif Finder	http://www.hprd.org/PhosphoMotif_finder
COBALT	http://www.st-va.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi?

4.1.3. Experimental Animal

For all experiments, *Hydra vulgaris AEP* animals were used.

4.1.4. Bacterial strains

Strain	Supplier
E. coli DH5α	Invitrogen
JM109	Promega

4.1.5. Vector

Name	Supplier
pGEM®-T Easy Vector	Promega

4.1.6. Oligonucleotide

All oligonucleotides were purchased from Sigma – Aldrich

Name	Sequence	Tm^0
Dof (F)	5'-GTTGCAGTTTTTAATTCAAATATACC-3'	59.7
Dof (R)	5'-TTGCAGCTGCTATGTCCATTGG-3'	69.2
FRS2 (F)	5'-ATGGAGGTAATTTTGGAAGGC-3'	62.8
FRS2 (R)	5'-GACCTACTACATTCAAATCGA-3'	55.8
Spry1 (F)	5'-ATGTATAGTGTGCGTGAAAGCG-3'	63.8
Spry1 (R)	5'-CCATAGGGAAAAGAAGTCGGG-3'	65.5
Spry2 (F)	5'-CAACCAGCATATCCAAAAGACC-3'	64.3
Spry2 (R)	5'-GTGTCTTTTTTTCCTTTTAACTCG-3'	60.9
Spry3 (F)	5'-AGCCTAAATGTTCGTGTTCGC-3'	65.1
Spry3 (R)	5'-GGTAGCAACTTAGACAGGGG-3'	60.2
Spry4 (F)	5'-ATGGATAAGCAAACACACGAAT-3'	62.1
Spry4 (R)	5'-TACAACAGTCTAAAACGCCATTC-3'	61.8

4.1.7. DNA and RNA size standards

Name	Supplier
DNA- Marker: O'GeneRuler DNA ladder mix	Fermentas
RNA-Marker: RiboRuler RNA ladder	Fermentas
Proteins-Marker: Protein Molecular Weight Marker	Fermentas

4.1.8. Enzyme

Name	Supplier
Reverse Transcriptase	Fermentas
DNA-Polymerase	Fermentas
Proteinase K	Roche
Rastriction enzyme	Fermentas
T7 RNA-Polymerase	Roche
SP6 RNA-Polymerase	Roche
Taq DNA-Polymerase	Fermentas

4.1.9. Antibody and Serum

Name	Supplier
Anti-Digoxigenin-AP Fab-Fragment	Roche
Horseradish peroxidase coupled anti-rabbit IgG	Dianova
Alkaline phosphatase-coupled anti-rabbit IgG	Dianova
FKS	PAA
Sheep serum	Sigma

4.1.10. Chemicals

All standard chemicals which are not mentioned were purchased from Roth, Serva, Merck and Sigma based on p.a. quality.

Name	Supplier
Blocking Reagent	Roche
BM Purple	Roche
Fast Red tablet	Roche
NBT/BCIP	Roche
BSA Fraction V, pH 7, standard grade, lyophilisied	Serva
Euparal	Roth

Transcription buffer (10X)	Roche
Dig-Labelling mix (10X)	Roche
Digoxigenin-11-UTP	Roche
SU5402 (FGFR inhibitor)	Calbiochem
UO126 (MEK inhibitor)	Calbiochem
RNA loading buffer	Fermentas

4.1.11. Ready kits

Name	Supplier
QuickPrep Micro mRNA Purification Kit	Amersham
QIAprep Spin Miniprep Kit	Qiagen
pGEM®-T Easy Vector Systems	Promega
RevertAid™ Premium First Strand cDNA Synthesis Kit	Fermentas
SMARTer™ RACE cDNA Amplification Kit	Clontech Laboratories
Advantage® 2 PCR Kit	Clontech Laboratories

4.1.12. Special materials

Name	Supplier
Protran& Nitrocellulose transfer membrane (0.45 µm)	Schleicher & Schuell
Whatman-Blotting Paper (3 mm)	Whatman-Biometra
X-ray film : Hyperfilm	Amersham
Biodyne B-Membrane	Pall
Microcon Centrifugal Filter Devices YM-10	Millipore

4.1.13. Special equipment

Name	Supplier
Fluorescence microscopy (NikonTS2000 Eclipse)	Nikon
Leica confocal laser scanning microscope (TCS SP2 and SP5)	Leica
Semi-Dry blot apparatus	Cti

4.1.14. Solutions and media

All media and solutions were prepared with MilliQ water

4.1.14.1. Culture medium for *Hydra vulgaris* AEP:

0.29 mM $CaCl_2$

0.59 mM $MgSO_4$
0.5 mM $NaHCO_3$
0.08 mM K_2CO_3
Adjust pH 7.4

4.1.14.2. Solutions for *Hydra* experiments

Dissociation medium:	3.6 mM KCl
	6 mM CaCl x $2H_2O$
	1,2 mM $MgSO_4$ x $2H_2O$
	6 mM Na_3-Citrate
	6 mM Na-Pyruvate
	6 mM Glucose
	12.5 mM TES
	0.05 mg/ml Rifampicin
	in *Hydra* medium, pH 7.4
Maceration solution:	glycerol: glacial acetic acid: water at a ratio 1:1:13

4.1.14.3. Media for bacterial work

LB- Medium	10 g Bacto-tryptone
	5 g Bacto-yeast extract
	5 g NaCl
	Adjust pH to 7.0 with NaOH. Sterilize by autoclaving.
SOC medium	2.0 g Bacto-tryptone
	0.5 g Bacto-yeast extract
	1 ml 1 M NaCl
	0.25 ml 1 M KCl
	1ml 2 M Mg^{2+} stock, filter-sterilized
	1ml 2 M glucose, filter-sterilized
2M Mg^{2+} stock	20.33g $MgCl_2 \cdot 6H_2O$
	24.65g $MgSO_4 \cdot 7H_2O$
	Add distilled water to 100ml. Filter Sterilized.

Selection agents, final concentrations	
Ampicilin	50 µg/ml
IPTG	0,5 mM
X-Gal	40 µg/ml

4.1.14.4. Solutions for gel electrophoresis

TBE (10X Stock solution)	0.9 M Tris-Boric acid 20 mM EDTA pH 8.2
TE (1X)	10 mM Tris-HCl pH 7.6 1 mM EDTA

4.1.14.5. Solutions for Northern blot

MOPS buffer	200 mM MOPS 50 mM Sodium acetate 10 mM EDTA pH 7
Wash buffer	100 mM Tris pH 7.5 150 mM NaCl DEPC water
Stain marker	0.02% Methylene blue 0.3 M Sodium acetate pH 5.5
Staining buffer	100 mM Tris pH 9.5 100 mM NaCl 50 mM $MgCl_2$
Blocking buffer	Blocking reagent for nucleic acid hybridization and detection in maleic acid buffer as recommended by manufacturer

4.1.14.6. Solutions for *in situ* Hybridization

For working with RNA all solutions were prepared in autoclaved DEPC water.

DEPC-H_2O	0,1% Diethylpyrocarbonate in MilliQ H_2O Overnight incubated at RT and autoclaved
Blocking Solution	80% MAB-B 20% sheep serum
50 X Denhardt's	1% polyvinylpyrrolidone 1% Ficoll 1% BSA fraction V in MilliQ H_2O
SSC (20X)	3 M NaCl 0.15 M Sodium citrate
Hybridization solution	50% Formamide 5 x SSC 200 μg/ml torula RNA 0,1% Tween 20 0,1% CHAPS 1X Denhardt's 100 μg/ml Heparin in DEPC water
MAB	100 mM Maleic acid 150 mM Nacl pH 7.5
MAB-B	MAB + 1% BSA
NTMT	100 mM NaCl 100 mM Tris-HCl, pH 9.5 50 mM $MgCl_2$

	0.1% Tween
Fixative	4% Paraformaldehyde (PFA) in *Hydra* medium
PBS	150 mM NaCl 80 mM Na_2HPO_4 21 mM NaH_2PO_4 pH 7,34
PBT	PBS + 0.1% Tween
Proteinase K stock solution	10 mg / ml proteinase K in DEPC water
Urethane (2%)	dissolved in *Hydra* medium

4.1.14.7. Solutions for protein analysis

Alkaline phosphatase buffer	100 mM Tris-HCl, pH 9.5 5 mM $MgCl_2$ 100 mM NaCl
Blocking solution	45 ml 1 x PBS 5 ml FKS (Fetal calf serum) 0.25 ml Tween 20 0.5 g BSA (Bovine serum albumin)
ECL-A	200 ml 0.1M TRIS-HCl (pH 8.6) 50 mg Luminol
ECL-B	11 mg para-Hydroxycoumaric acid in 10 ml DMSO
ECL working solution	1 ml ECL-A 100 µl ECL-B 0.3 µl H_2O_2 (30%)

Laemmli running buffer (10X)	250 mM Tris-HCl, pH 8.3 1,92 M Glycin 1% SDS
Laemmili sample buffer (2X)	2% SDS 20% glycerol 2% β-Mercaptoethanol 20 mM Tris-HCl, pH 6.8 0.04 M Bromphenolblue
Laemmli-Separating gel buffer	1.5 M Tris-Cl pH 8.8 0.4% SDS
Laemmli-Stacking gel buffer	1.5 M Tris-Cl, pH 6.8 0.4% SDS
PBS (10X)	87.7 g NaCl in 1l 0.1 M phosphate buffer
Phosphate buffer (0.1 M)	0,1 M Na_2HPO_4 0,1 M NaH_2PO_4 Mix until pH reaches 7.4
Ponceau-Staining solution	0.2% Ponceau in 3% TCA
Stock solution I (Coomassie)	5% Coomassie Brilliant Blue G250 in dH_2O
Stock solution II (Coomassie)	2% (w/v) Phosphoric acid 10% NH_4SO_4 in dH_2O
Working staining solution (Coomassie)	0.1% Stock solution I in Stock solution II

4.2. Methods

4.2.1. Bioinformatic analysis

4.2.1.1. Database search

To reveal the Dof, FRS2, Grb2, Shp2 and Spry sequences in *Hydra*, we explored the NCBI, JGI, hydrazome/ metazome, Compagen T-CDS: Transcript models (contigs) derived from assembled ESTs (Hemmrich and Bosch 2008) and RNASeq project (Wenger and Galliot 2013). Predicted protein sequences for Dof, FRS2, Grb2, Shp2, and Spry were further analyzed for conserved domains using NCBI's conserved domain search tool (Marchler-Bauer et al. 2015), ExpasyProsite, Pfam, Motif scan (Pagni et al. 2007) and PhosphoMotif finder (Amanchy et al. 2007). DOG 1.0 software was used to draw conserved domain figure (Fig. 6, 11, 17, 18 and 19) (Ren et al. 2009).

4.2.1.2. Phylogenetic analysis

4.2.1.2.1. Dof

Predicted *Hydra* Dof protein sequences were aligned with Dof protein sequences from the insects *Drosophila melanogaster* and *Tribolium castaneum*, the molluscs *Crassostrea gigas* and *Lottia gigantea*, the annelid *Capitella teleta*, the hemichordate *Saccoglossus kowalevskii*, the echinoderm *Strongylocentrotus purpuratus* and *Homo sapiens* using ProbCons version 1.12 (Do et al. 2005), clustalX, COBALT (Papadopoulos and Agarwala 2007) programs. Databases and accession numbers are indicated in supplement Fig. S1. Alignments were analyzed in Jal view version 2.8 (Waterhouse et al. 2009) and manually edited in Genedoc (for alignments see Fig. S2 and S3). Phylogenetic trees were calculated; using the conserved DBB domain together with ankyrin repeats and as well as using the whole sequence. For all trees, sequence gaps were deleted. See Fig. S4 for sequences used for phylogenetic tree.

4.2.1.2.2. FRS2

For phylogenetic analysis of FRS2 proteins, a multiple sequence alignment of the predicted *Hydra* FRS2 and the anthozoan *Nematostella vectensis* FRS2 was performed using FRS2 sequences of the choanoflagellate *Salpingoeca rosetta*, the sponge *Amphimedon queenslandica*, the parazoan *Trichoplax adhaerens.* As

representatives of the Protostomia, several lophotrochozoan FRS2 sequences were added: the platyhelminths *Clonorchis sinensis* and *Echinococcus granulosus*, the molluscs *Crassostrea gigas* and *Lottia gigantea*, the annelids *Capitella teleta* and *Helobdella robusta* as well as those of several ecdysozoans: the nematode *Caenorhabditis elegans*, the insects *Tribolium castaneum* and *Drosophila melanogaster*. As deuterostomian representatives, FRS2 of the hemichordate *Saccoglossus kowalevskii*, the urochordate *Ciona intestinalis* and the chordates *Danio rerio Xenopus laevis, Mus musculus* and *Homo sapiens* were integrated. A multiple sequence alignment was calculated with ProbCons version 1.12, clustalX, COBALT, T-coffee version 8.99 (Notredame et al. 2000) and MAFFT L-INS-i version 7.037b (Katoh and Standley 2013) with default settings. Subsequently, data were combined into an optimal alignment using the combiner function of T-coffee, Jalview version 2.8 (Waterhouse et al. 2009). InterProScan5 was used to visualize and analyze the alignments whereas Genedoc was used to manually edit alignments (for alignments see Fig. S5). Phylogenetic trees were constructed; first, with conserved PTB and PH domains and later also using the whole sequences. Sequence gaps were deleted for all trees. Databases and accession numbers are indicated in supplementary Fig. S1. For sequences used for the phylogenetic tree refer to Fig. S6.

4.2.1.2.3. Spry

Sequences of predicted *Hydra* Spry proteins were aligned with *Nematostella vectensis, Drosophila melanogaster, Tribolium castaneum, Culex quinquefasciatus, Aedes aegypti, Crassostrea gigas, Lottia gigantea, Aplysia californica, Capitella teleta, Helobdella robusta, Ascaris suum, Saccoglossus kowalevskii, Strongylocentrotus purpuratus, Paracentrotus lividus, Ciona intestinalis, Oikopleura dioica, Branchiostoma lanceolatum, Homo sapiens, Mus musculus, Danio rerio, Xenopus laevis*. Alignment was performed using ProbCons version 1.12 (Do et al. 2005), clustalX, COBALT (Papadopoulos and Agarwala 2007), T-coffee version 8.99 (Notredame et al. 2000) programs with default settings. Jal view version 2.8 (Waterhouse et al. 2009) used for analysis of alignments and manually edited in Genedoc (for alignments see Fig. S16). Two phylogenetic trees were calculated. The first using the conserved Cbl TKB binding motif, the RBD (Raf1-binding domain), the Spry domain (Fig. S18), and

the second using the whole sequences (Fig. 21). Databases and accession numbers are as indicated in Fig. S14.

4.2.1.2.4. Tree calculation

For the phylogenetic tree of Dof and FRS2: WAG + G + I model and for Spry: JTT + G + I model was selected as the best fitting amino acid substitution model according to the Bayesian Information Criterion in ProtTest version 3.3 (Darriba et al. 2011). Phylogenetic trees were calculated using Mr Bayes 3.1.2 (Huelsenbeck and Ronquist 2001). We initiated two runs of four Markov-chain Monte Carlo (MCMC) chains of 2×10^7 generations, each from a random starting tree. Sampling made every 1,000 generations [additional settings for Dof and FRS2: rates = invgamma, ngammacat = 4, aamodelpr = WAG. additional settings for Spry: rates = invgamma, ngammacat = 4, aamodelpr = JTT]. A 25% burn-in was selected, and convergence was assessed by a standard deviation of split frequencies falling below 0.005. The resultant trees were visualized with Figtree version 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

4.2.1.3. Protein 3D structure prediction

Three-dimensional structure of Spry protein (Fig. 20) was predicted by i-Tasser software (Roy et al. 2010). The SWISS-MODEL server was used for Protein Structure & Model Assessment (Biasini et al. 2014). The resultant structure was validated with the help of Ramachandran plot (RAMACHANDRAN et al. 1963).

4.2.1.4. Epitope prediction for the generation of antibody

Epitopes to generate an antibody against *Hydra* Spry were predicted using http://tools.immuneepitope.org/bcell/ from IEDB Analysis resources. Different methods such as Kolaskar & Tongaonkar Antigenicity (Kolaskar and Tongaonkar 1990), Chou & Fasman Beta-Turn Prediction (Chou and Fasman 1978), Emini Surface Accessibility Prediction (Emini et al. 1985), Karplus & Schulz Flexibility Prediction, Parker Hydrophilicity Prediction (Parker et al. 1986), Bepipred Linear Epitope Prediction (Larsen et al. 2006) were used to identify the best epitope. Predicted peptide sequences were analyzed through BLAST and the Expert Protein Analysis System proteomics server. Properties of predicted peptide were analyzed with the help of structure predictor, Hydrophobicity plotter, and Structure analyzer.

4.2.2. *Hydra* cultures

Hydra vulgaris AEP strain was cultured in a medium containing (0.29 mM $CaCl_2$, 0.59 mM $MgSO_4$, 0.5 mM $NaHCO_3$ and 0.08 mM K_2CO_3, pH 7.4) at 18°C. The animals were fed five times a week with freshly hatched *Artemia nauplii* to synchronize their growth (Sudhop et al. 2004).

4.2.3. Molecular biology methods

4.2.3.1. Isolation of mRNA

mRNA's were harvested from *Hydra vulgaris AEP* strain using Quickprep Micro mRNA Kit (Amersham) following the manufacturer's protocol and assuming that a mid-size *Hydra* polyp contains about $5x10^4$ cells.

4.2.3.2. Complementary DNA (cDNA) synthesis

For cDNA synthesis, 500 ng of mRNA per sample were used. RNA was reverse transcribed using Revert Aid TM Premium First-strand cDNA Synthesis Kit (Fermentas) following the manufacturer's protocol.

4.2.3.3. Polymerase Chain Reaction (PCR)

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed using cDNA as a template.

Reagent	Volume (µl)
10 x Taq standard PCR buffer	2 µl
dNTPs (10 mM each)	2 µl
$MgCl_2$ (25mM)	2 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Taq polymerase (0.5 U/µl)	0.2 µl
Template cDNA	2 µl
Water	fill to final volume
Total	20 µl

PCR program:

	Cycle	Temperature	Time
Denaturation	1	94°C	2 min
Annealing		50°C - 65°C ($^{+}$/$_{-}$ 5°C depend upon Tm of primers)	2 min
Extension		72°C	2 min
Denaturation	29	94°C	1 min
Annealing		50°C - 65°C ($^{+}$/$_{-}$ 5°C depend upon Tm of primers)	1 min
Extension		72°C	1 min
Final Extension	1	72°C	10 min
Hold		4°C	∞

4.2.3.4. Agarose gel electrophoresis

4.2.3.4.1. Agarose gel for DNA separation

The PCR products were mixed with DNA loading buffer and loaded onto 1.5 to 2% gels depending on the expected size of DNA. Gel electrophoresis performed in 1X TBE buffer for 30 min to 45 min at 120V. Then gels were stained with ethidium bromide (10µg/100ml) and visualized under UV light. DNA molecular weight standard used to determine the size of DNA fragments.

4.2.3.4.2. Agarose gel for RNA separation

RNA samples mixed with MOPS, formaldehyde, formamide and RNA loading buffer were incubated at 55^{0}C for 15 min. Samples were loaded in 1.5% Agarose gel containing MOPS and formaldehyde. Gels were run at 50mA for 1.5 to 2 hrs. Afterward, the gel was washed with DEPC water, stained and visualized under UV light.

4.2.3.5. Cloning

PCR products were ligated into the pGEM®-T Easy Vector following the manufacturer's protocol. JM109 High-Efficiency Competent Cells were transformed using 2 µl ligation reaction mixture and plated onto LB/Ampicillin/IPTG/X-Gal plates

as recommended by the manufacturer. White colonies (positive clones) were selected and cultured in LB-Amp$^+$ medium.

4.2.3.6. Plasmid DNA isolation

Plasmids were isolated and purified using the QIAprep spin Miniprep kit (Qiagen). Resultant plasmids were subjected to restriction digestion. The identity of the insert was confirmed by sequencing (SeqLab) and the plasmid used for Dig-labelled RNA probe synthesis via SP6 and T7 polymerase (Roche).

4.2.3.7. Insert PCR for probe synthesis

For probe synthesis or checking insert, a typical Insert PCR performed.

Reagent	Volume (µl)
10 x Taq standard PCR buffer	2 µl
dNTPs (10 mM each)	2 µl
$MgCl_2$ (25mM)	2 µl
SP6 primer (10 mM)	1 µl
T7 primer (10 mM)	1 µl
Taq polymerase (1 U/µl)	0.2 µl
Template DNA	2 µl
water	fill to final volume
Total	20 µl

Insert PCR program:

	Cycle	Temperature	Time
Denaturation	30	95°C	1 min
Annealing		50°C - 65°C ($^+/_-$ 5°C depend upon Tm of primers)	30 sec
Extension		72°C	3 min
Final Extension	1	72°C	10 min
Hold		4°C	∞

4.2.3.8. Probe synthesis

Sense and anti-sense riboprobes were synthesized (i.e. labeling RNA with digoxygenin-UTP) as follows:

Transcription reaction mixture:

10x transcription buffer	1 µl
10x Digoxygenin labelling mix	1 µl
Linearized DNA	1 µl
T7 or SP6 polymerase	1 µl
DEPC water	Adjust volume to 10 µl

Components were mixed and incubated at 37^0C for 3 hrs. RNA precipitated with LiCl and ethanol. Then RNA pellets washed and dissolved in DEPC water.

4.2.3.9. Northern blot

4.2.3.9.1. Preparation of Formaldehyde gel

Running buffer

10 x MOPS	35 ml
37 % formaldehyde	62.5 ml
DEPC-treated water	Adjust volume to 350 ml

Formaldehyde gel

DEPC-treated water	37 ml
Agarose	5 ml
10 x MOPS	0.4 g
Heated until agarose dissolved and cooled to 60^0 C	
37 % formaldehyde	9 ml
Mixed and poured into gel chamber. Allowed gel to set for 30 min	

Sample preparation and loading into gel

	Probe	RNA	Marker
DEPC Water	4,5 µl	/	2,5 µl
10x MOPS	1 µl	1 µl	1 µl
Formaldehyd	3,5 µl	3,5 µl	3,5 µl
Formamid	10 µl	10 µl	10 µl
Probe (1/10)	1 µl	7,5 µl	3 µl RNA Marker
Total Volume:	20 µl	20 µl	20 µl

Samples were loaded onto the gel and run gel at 50 mA for 2 hrs.

4.2.3.9.2. Setting up the Northern blot

Nucleic acids were transferred from the agarose gel to Hybond N membrane by the classical Upward Capillary Transfer method (Molecular cloning: a laboratory manual; Sambrook and Russell; Third Edition**)**.

4.2.3.9.3. Hybridization and blot development

After the transfer of nucleic acids to the membrane, the membrane was washed with washing buffer and dried in between Whatmann paper for 1 hr at 80^0C. RNA marker part cut out and stained with 0.02% methylene blue / 0.3 M Na Ac pH 5.5 until the marker bands are visible. Remaining membrane washed with washing buffer and blocked with blocking buffer for 1 hr. Again membrane washed with washing solution and incubated with Anti-Dig antibody solution (1/5000 dilution) for 30 min. Once again, membrane washed with washing buffer and stained with the staining solution until bands are visible.

4.2.3.10. *In situ* hybridization

Whole mount and single cell preparation *in situ* hybridization was performed as described in (Sudhop et al. 2004) (Bode, 1996; Grens et al., 1995; Lange et al., 2014). Bud stages were selected with reference to (Otto and Campbell 1977). The Dig-labeling system (ROCHE) was used to synthesize Digoxigenin-labelled RNA probes. *HAEP_dof RNA* probe (571bp) and *HAEP_FRS2 RNA* probe (561bp) were synthesized against nucleotides 1,496bp – 2,067bp and 166bp – 727bp respectively. *HAEP_spry1 RNA* probe (310bp), *HAEP_spry2 RNA* probe (423 bp), *HAEP_spry3 RNA* probe (447bp) and *HAEP_spry4 RNA* probe (729bp) were synthesized against nucleotides (690bp -972bp), (676bp – 1098bp), (271bp - 718bp) and (1bp - 729bp) respectively. The quality of RNA probes was verified by Northern blotting and about 300 ng of RNA probes were used for the *in situ* hybridization.

4.2.3.11. Smarter RACE

RACE-Ready First-strand cDNA synthesis and 5'- and 3'-RACE PCR reactions were carried out according to manufacturer's protocol.

4.2.3.12. Western blot

Specificity of Anti-Spry2 antibody was determined by western blot. Three *Hydra* polyps were boiled for 2min in 25 µl standard loading buffer (2X Laemmli buffer). Then samples were immediately cooled on ice and subject to denaturing PAGE. A Western blot was performed according to standard procedures on a PVDF membrane. The primary antibody (rabbit polyclonal anti-Spry antibody, Davids Biotechnologie) was used 1:200 diluted in PBS/0.005% Tween 20 with 0.1% FKS and 0.01% Bovine serum albumin (BSA fraction V). As a secondary antibody, peroxidase-coupled anti-rabbit antibody (Dianova) was used and detection was carried out using ECL system.

4.2.3.13. Immunohistochemistry

4.2.3.13.1. Whole mount immunohistochemistry

Hydra vulgaris AEP animals were relaxed in 2% urethane for 1 min at 18^0C. They were fixed overnight in 4% paraformaldehyde in 1X PBS pH 7.4. Then animals were washed and permeabilized 3 x15 min in PBT (1X PBS with pH 7.4 and 0.25% Triton x-100, v/v). Polyps were incubated overnight in blocking solution (1X PBT containing 10% FKS). Primary antibody (Rabbit polyclonal anti-spry antibody, Davids Biotechnologie) was incubated at 40^0C overnight with dilution 1:50 in blocking buffer. After that 1X 5min, 10 min, 30min washing in PBT at room temperature. Following specimens were incubated in secondary antibody (Anti-Rabbit IgG (whole molecule)-FITC) for 2 hrs at room temperature. Samples without primary antibody were used as a control. Unbound antibody was removed by washing specimens 3x15 min in PBT followed by 2x15 min in PBS, pH 7.4 at room temperature. Specimens were embedded in Roti®-Mount FluorCare (Carl Roth) and stored in the dark at 4^0C. Staining was examined by fluorescence microscopy (NikonTS2000 Eclipse) and Leica confocal laser scanning microscope (TCS SP2 and SP5). Leica LAS software and Adobe Photoshop CS2 software were used to process pictures.

4.2.3.13.2. Macerate Immunohistochemistry

In the case of Macerate Immunohistochemistry, single cell preparation was made as per (Sudhop et al. 2004). The whole mount Immunohistochemistry protocol was followed with these single cell preparation slides.

4.2.3.14. Inhibitor study

4.2.3.14.1. MEK inhibitor (UO126) and FGFR inhibitor (SU5402)

UO126 specifically inhibits MEK1 with IC_{50} of 74 and MEK2 with 58 nM. Animals were treated with UO126 and SU5402 as per given in (Hasse et al. 2014).

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6. Appendix A- Abbreviation

aa	amino acid
Amp	Ampicillin
AP	Alkaline Phosphatase
bp	Base pair
BCIP	5-Bromo-4-chloro-3-indolylphosphate
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
Da	Dalton
DAG	Diacylglycerol
dATP	Deoxy Adenosine nucleotide triphosphate
dCTP	Deoxy Cytosine nucleotide triphosphate
DEPC	Diethylpyrocarbonate
ddH2O	Double distilled water
DIG	digoxygenin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EtBr	Ethidiumbromide
FCS	fetal calf serum
HCl	hydrogen chloride
HRP	horseradish peroxidase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISH	in situ hybridization
kb	Kilo base pair
kDa	Kilo dalton

KCl	potassium chloride
LB	lysogeny broth
LB^{AMP}	LB medium with 100 µg/ml Ampicillin
LiCl	Lithium-Chloride
M	Molar
mA	Mili Ampere
mg	milligram
ml	millilitre
mM	millimolar
MAB	maleic acid buffer
MgCl2	magnesium chloride
NaCl	sodium chloride
NaHCO3	sodium bicarbonate
NaOH	sodium hydroxide
NBT	nitro blue tetrazolium
NTMT	sodium-tris-magnesium-Tween 20
OD	optical density
oligo	oligonucleotide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PVDF	polyvinylidene fluoride
RACE	Rapid Amplification of cDNA Ends
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SSC	saline-sodium citrate
TAE	tris-acetate-EDTA
Taq	Thermus aquaticus
TBE	tris-borate-EDTA

TEA	triethanolamine
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
Tween 20	Polyoxyethylene (20) sorbitan monolaurate

7. Appendix B- Supplements

S1: Names and database accession numbers of Dof and FRS2 sequences
S2: Alignment of Dof proteins excluding Acropora millepora Dof used to calculate the phylogenetic trees and conserved domain figure
S3: Alignment of Dof proteins including Acropora millepora Dof used to calculate the phylogenetic trees and conserved domain figure
S4: All Dof sequences used for the phylogenetic tree
S5: Alignment of FRS2 proteins used to calculate the phylogenetic trees and conserved domain
S6: All FRS2 sequences used for the phylogenetic tree
S7: Names and database accession numbers of all used IRS sequences
S8: All IRS sequences used for the alignment and phylogenetic tree
S9: Names and database accession numbers of Dok sequences
S10: All Dok sequences used for the alignment and phylogenetic tree
S11: Alignment of IRS proteins with FRS2 proteins
S12: Alignment of FRS2, PH-FRS2, IRS and Dok proteins
S13: Names and database accession numbers of Grb2 and Shp2 sequences
S14: Names and database accession numbers of Spry sequences
S15: Search for Spry protein in *Hydra*: experimental approaches
S16: Alignments of Spry proteins used to calculate the phylogenetic trees and conserved domain figure
S17: All Spry sequences used for alignment and phylogenetic tree
S18: Phylogenetic tree of conserved domains in Sprouty proteins
S19: Gel images of *Hydra* AEP cDNAs amplified by PCR
S20: Expression pattern of *spry1* and *spry4* mRNA
S21: RNA northern blot for checking endogenous antisense RNA
S22: *spry1* sequence analysis for ribosomal RNA
S23: Expression pattern of *spry3* mRNA

S24: Anti-Spry2 Antibody information

S25: Calibration curve including value table

S26: Search for Dof full length sequence

S27: Search for FRS2 full length sequence

S28: Grb2 sequences

S29: Shp2 sequence

8. Curriculum vitae

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Education:

2003:	Higher Secondary School Certificate (HSC) at Shivaji University, Maharashtra, India
2003-2008:	Bachelor of Pharmacy at Satara College of Pharmacy, Shivaji University, Maharashtra, India Grade: First class
2008-2010:	Master of Science in Bioinformatics at Sikkim Manipal University, India Grade: A, Remark: Excellent
Since 2011:	Doctoral thesis in the group of Prof. Dr. Monika Hassel at the Department of Biology, Morphology and Evolution of Animals, Philipps University, Marburg, Germany.

Manuscripts under preparation:

Isolation and expression analysis of genes encoding potential docking proteins for *Hydra* FGFR

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Sprouty2, a negative regulator of RTK signaling is coexpressed with both FGFRs at the *Hydra* bud base

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Poster:

Suryawanshi A., Heller J., Hassel M.
"First clue about Sprouty: A novel antagonist of FGFR signaling in *Hydra*". Presented at: 9th German Society for Developmental Biology (GfE) School "Regulatory Networks in Development"; 2012 September 13-15, Günzburg, Germany.

Suryawanshi A., Heller J., Hassel M.
"Sprouty proteins in *Hydra* -potential antagonist(s) of FGFR signaling?". Presented at: International Joint Meeting of the German Society for Cell Biology (DGZ) and the German Society for Developmental Biology (GfE); 2013 March 20-23, Heidelberg, Germany.

Suryawanshi, A., Rebscher, N., Heller, J., Westermann, J., Holz, O., Lange, E. and Hassel, M.
"Evidence for Sprouty and Dof, potential elements of FGFR signaling, in *Hydra*". Presented at: International Workshop, "Unravelling the Developmental Regulatory Network in Early Animals"; 2013 September 23-26, Evangelische Akademie Tutzing, Germany.

Marburg, November 2016

Ashwini Suryawanshi

Fig. S1: Names and database accession numbers of all used DOF and FRS2 sequences

Phylum	Species	Name of the DOF	Accession numbers	Full length	Database for DOF
Cnidaria	*Hydra vulgaris AEP*	HAEP_DOF	HAEP_TCDS_v02_8498	Yes	Compagen
	Hydra magnipapillata	Hm_DOF	Hma2.234406	Yes	Compagen Compagen Compagen Compagen
	Nematostella vectensis				GenBank GenBank
	Acropora millepora	Ami_DOF	Cluster003282	Yes	Prof. Dr. David Miller
Choanoflagellate	*Salpingoeca rosetta*				GenBank
Porifera	*Amphimedon queenslandica*				GenBank
Placozoa	*Trichoplax adhaerens*				GenBank
Platyhelminthes	*Clonorchis sinensis*				GenBank
	Echinococcus granulosus				GenBank
Mollusca	*Crassostrea gigas*	Cg_DOF_1 Cg_DOF_2 Cg_DOF_3	EKC18782.1, EKC42036.1, EKC18779.1	Yes	GenBank
	Lottia gigantea	Lg_DOF_1 Lg_DOF_2	ESP03804.1 ESO87157.1	Yes Yes	GenBank
Annelida	*Capitella teleta*	Ct_DOF	ELU09260.1	Yes	GenBank GenBank
	Helobdella robusta				GenBank
Insecta	*Tribolium castaneum*	Tr_Stumps	EEZ97730.1	Yes	GenBank
	Drosophila melanogaster	Dm_DOF	CAA09298.1	Yes	GenBank
	Anopheles gambiae	Ag_DOF	CAD27761.1	Yes	
Nematoda	*Caenorhabditis elegans*				GenBank
Echinodermata	*Strongylocentrotus purpuratus*	Sp_DOF	XP_003723617.1	Yes	GenBank
Hemichordata	*Saccoglossus kowalevskii*	Sk_DOF	XP_006814050.1	Yes	GenBank
Urochordata	*Ciona intestinalis*			Yes	GenBank
Vertebrata	*Homo sapiens*	Hs_DOF Hs_BANK Hs_BCAP	NP_689522.2 BAB79255.1 NP_001120979.2	Yes Yes Yes	GenBank
	Mus musculus				GenBank
	Danio rerio	Dr_DOF	XP_005156973.1	Yes	GenBank
	Xenopus laevis	Xe_DOF	XP_002936021.2	Yes	GenBank

Phylum	Species	Name of the FRS2	Accession numbers	Full length	Database for FRS2
Cnidaria	*Hydra vulgaris AEP*	HAEP_FRS2	HAEP_T-CDS_v02_13833	5'end missing	Compagen
	Hydra magnipapillata	Hm_FRS2_1	Hma2.207102	Yes	Compagen
		Hm_FRS2_2	Hma2.207103	Yes	Compagen
		Hm_FRS2_3	Hma2.207104	Yes	Compagen
		Hm_FRS2_4	Hma2.207105	Yes	Compagen
	Nematostella vectensis	Nv_FRS2_L1	XP_001635403.1	Yes	GenBank
		Nv_FRS2_L2	XP_001641972.1	Yes	GenBank
Choanoflagellate	*Salpingoeca rosetta*	Sro_FRS2	XP_004995975.1	Yes	GenBank
Porifera	*Amphimedon queenslandica*	Aqu_FRS2	XP_003383468.1	Yes	GenBank
Placozoa	*Trichoplax adhaerens*	Tad_FRS2	XP_002109860.1	Yes	GenBank
Platyhelminthes	*Clonorchis sinensis*	Cs_FRS3	GAA49670.1	Yes	GenBank
	Echinococcus granulosus	Egr_FRS2	CDJ19779.1	Yes	GenBank
Mollusca	*Crassostrea gigas*	Cg_FRS3	EKC36283.1	Yes	GenBank
	Lottia gigantea	Lg_FRS2	ESO92723.1	Yes	GenBank
Annelida	*Capitella teleta*	Ct_FRS2_L1	ELU17348.1	Yes	GenBank
		Ct_FRS2_L2	ELU09839.1	Yes	GenBank
	Helobdella robusta	Hro_FRS2	ESO04972.1	Yes	GenBank
Insecta	*Tribolium castaneum*	Tr_FRS2_L	XP_973347.1	Yes	GenBank
	Drosophila melanogaster	Dm_FRS2_L	AAM48392.1	Yes	GenBank
	Anopheles gambiae				
Nematoda	*Caenorhabditis elegans*	Ce_ROG-1	BAF48662.1	Yes	GenBank
Echinodermata	*Strongylocentrotus purpuratus*	Sp_FRS2	XP_781457.1	Yes	GenBank
Hemichordata	*Saccoglossus kowalevskii*	Sk_FRS2	XP_006823990.1	Yes	GenBank
Urochordata	*Ciona intestinalis*	Ci_FRS3	XP_002121024.1	Yes	GenBank
Vertebrata	*Homo sapiens*	Hs_FRS2	AAH21562.1	Yes	GenBank
	Mus musculus	Mm_FRS2	NP_808466.1	Yes	GenBank
	Danio rerio	Dr_FRS2	AAI65296.1	Yes	GenBank
	Xenopus laevis	Xe_FRS2	BAB61837.1	Yes	GenBank

Fig. S3: Alignment of Dof proteins including Acropora millepora Dof (A)

Fig. S5: Alignment of FRS2 proteins used to calculate the phylogenetic trees and Conserved domain figure (A) Only conserved domains alignment, (B) Whole sequence alignment. The blue bar indicates localization of the PTB domain.

A)

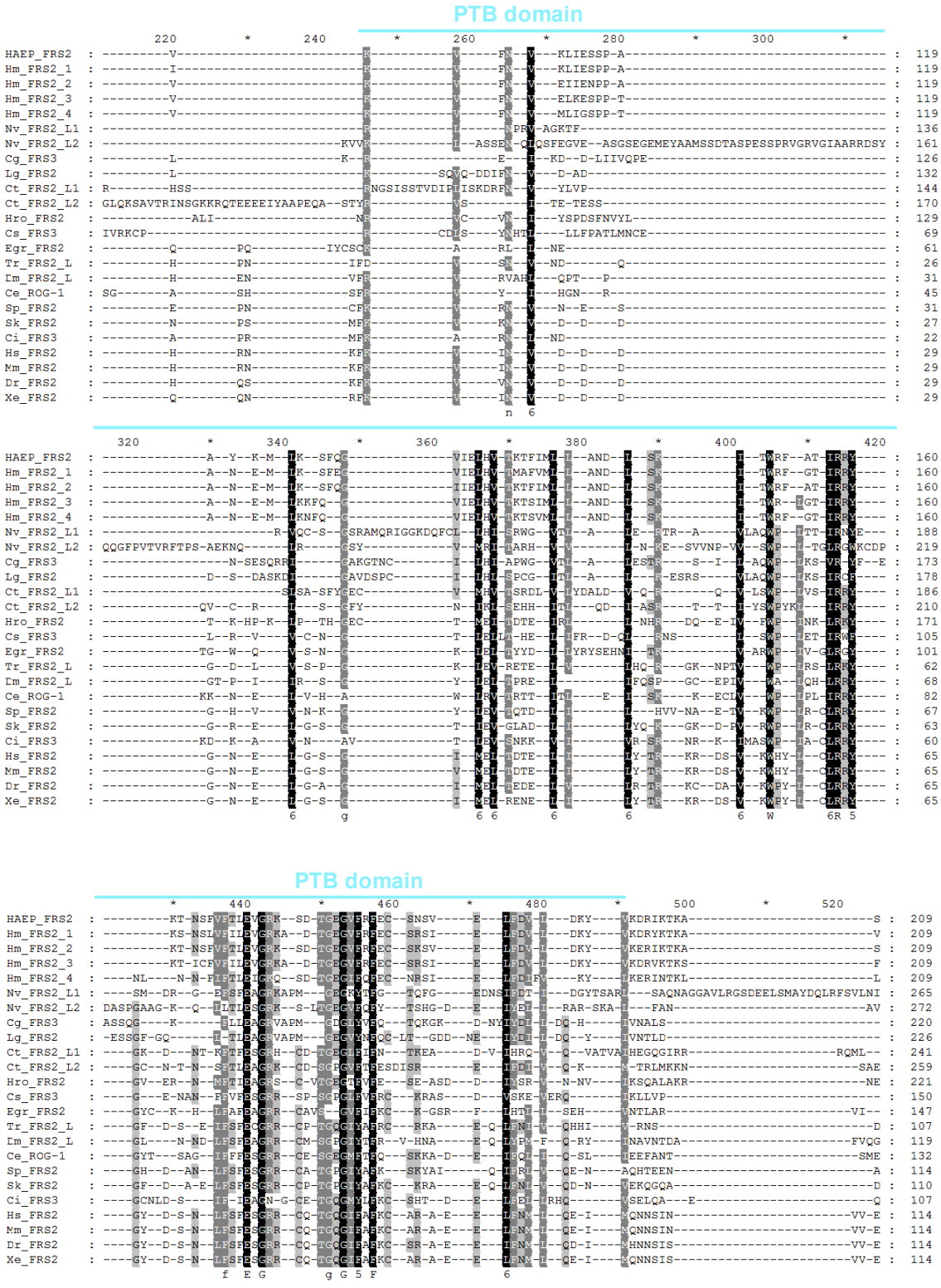
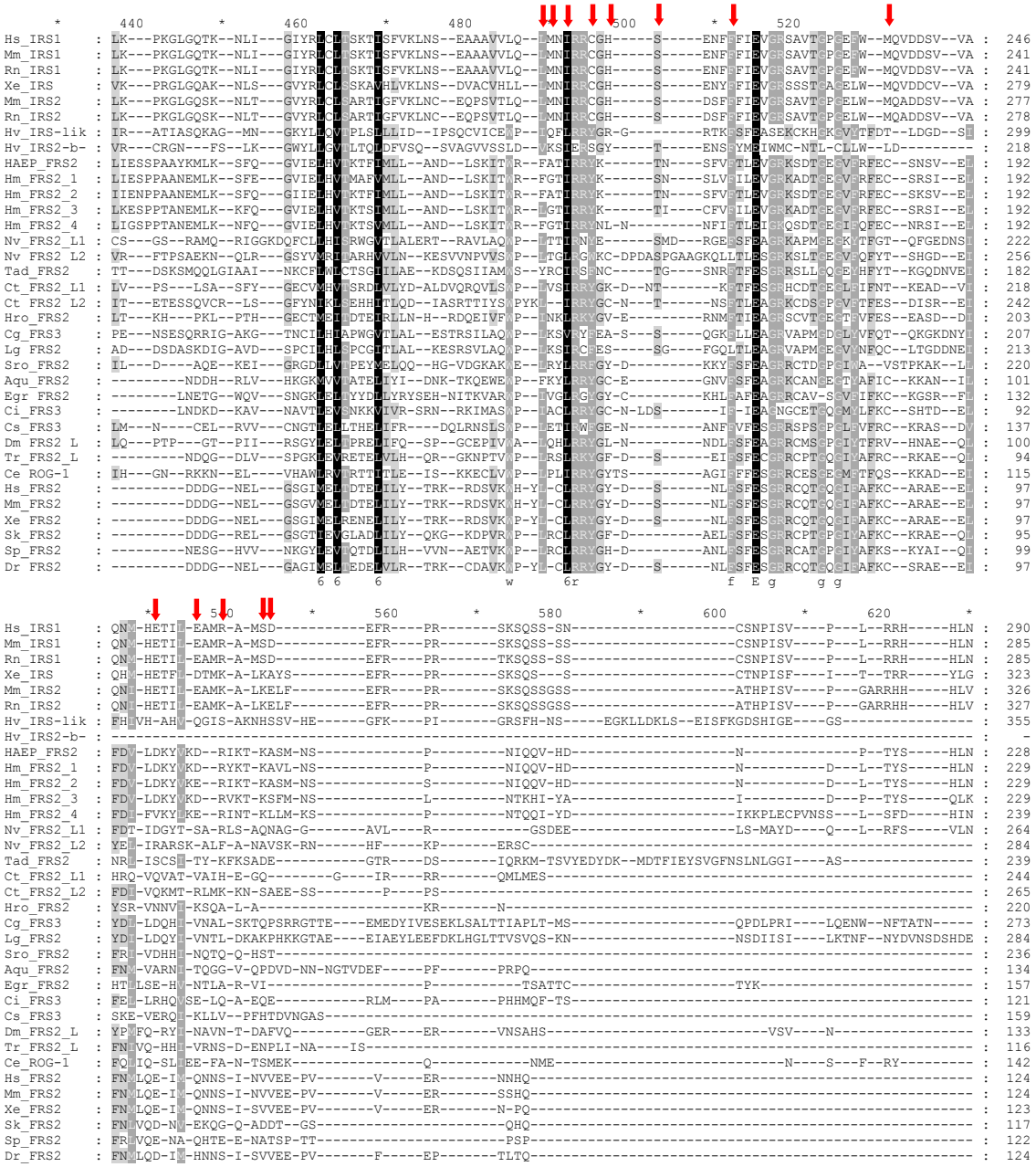


Fig. S7: Names and database accession numbers of all used IRS sequences

Phylum	Species	Name of the IRS	Accession numbers	Database
Cnidaria	*Hydra vulgaris*	Hv_IRS2-b-like	XP_004208803.1	GenBank
		Hv_IRS-like	NP_001267774.1	GenBank
	Nematostella vectensis	Nv_IRS-hyp	EDO47652.1	GenBank
Insecta	*Drosophila melanogaster*	Dm_IRS	AAD43005.1	GenBank
Vertebrata	*Homo sapiens*	Hs_IRS-1	NP_005535.1	GenBank
		Hs_IRS-2	NP_003740.2	GenBank
		Hs_IRS-4	NP_003595.1	GenBank
	Mus musculus	Mm_IRS1	NP_034700.2	GenBank
		Mm_IRS2	NP_001074681.1	GenBank
	Rattus norvegicus	Rn_IRS1	NP_037101.1	GenBank
		Rn_IRS2	NP_001162104.1	GenBank
	Xenopus laevis	Xe_IRS	AAG18441.1	GenBank

Fig. S11: Alignment of IRS proteins with FRS2 proteins. Red color arrow indicates residues needed for interaction of IRS-1 proteins with insulin receptor



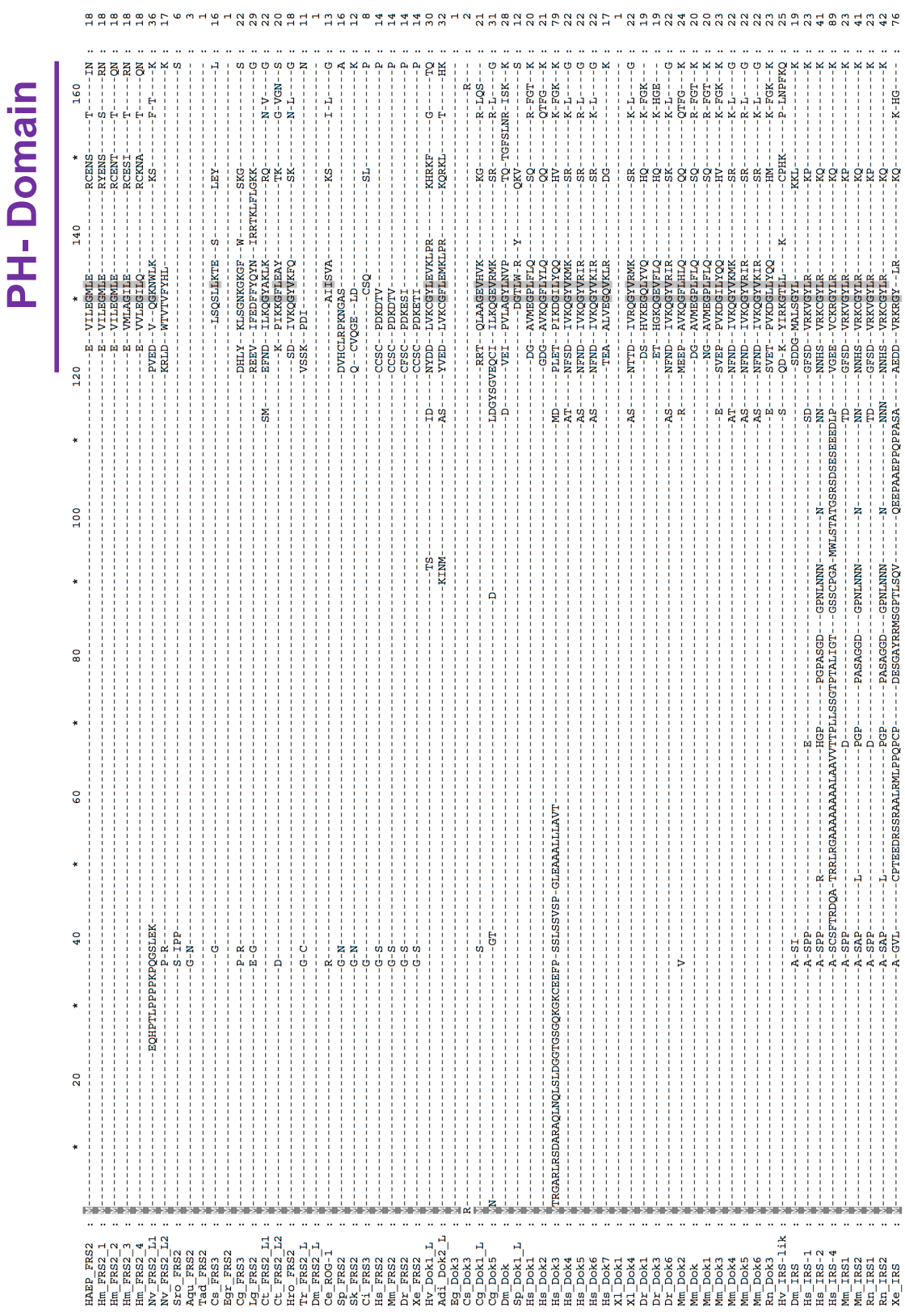
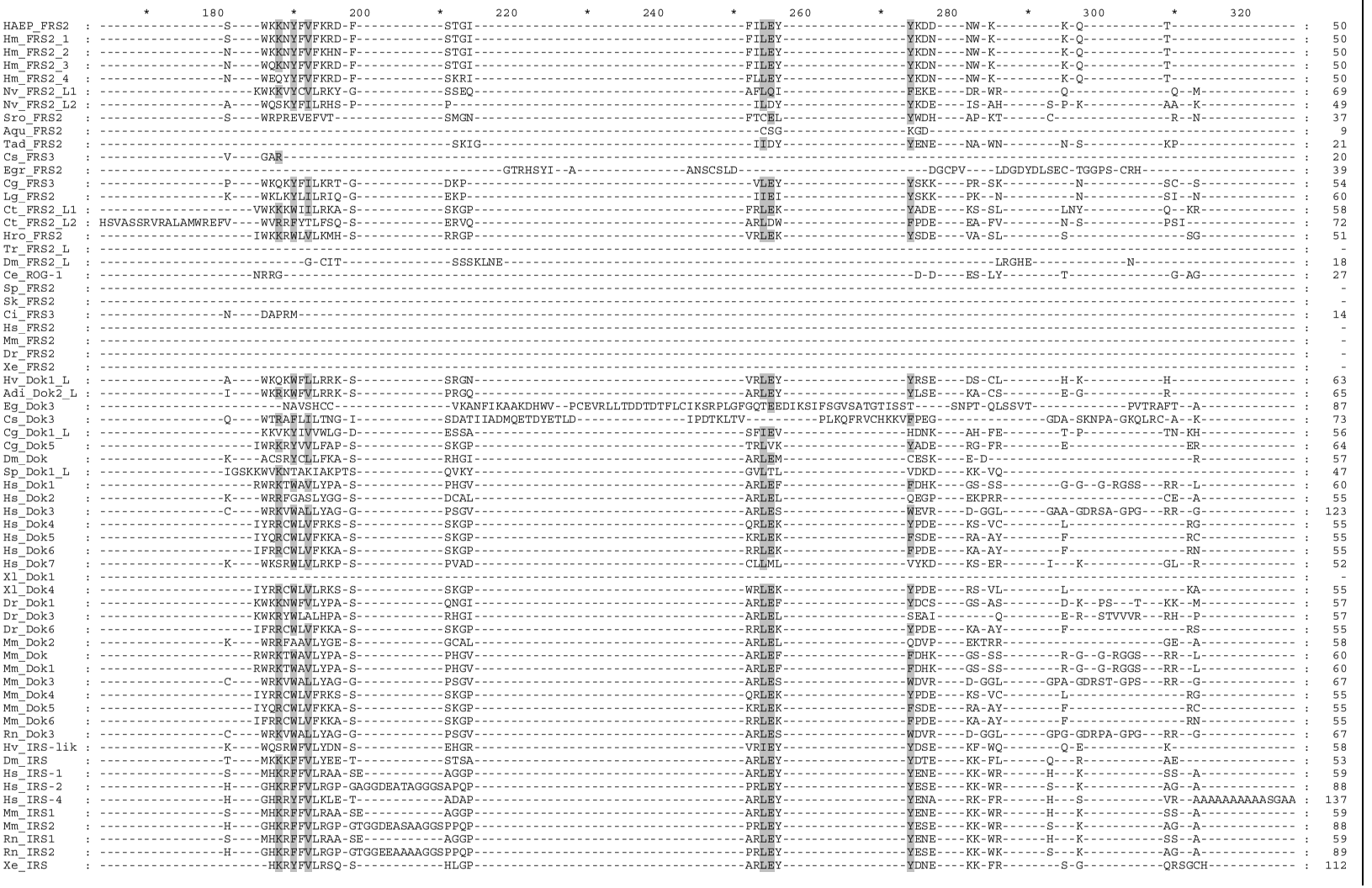
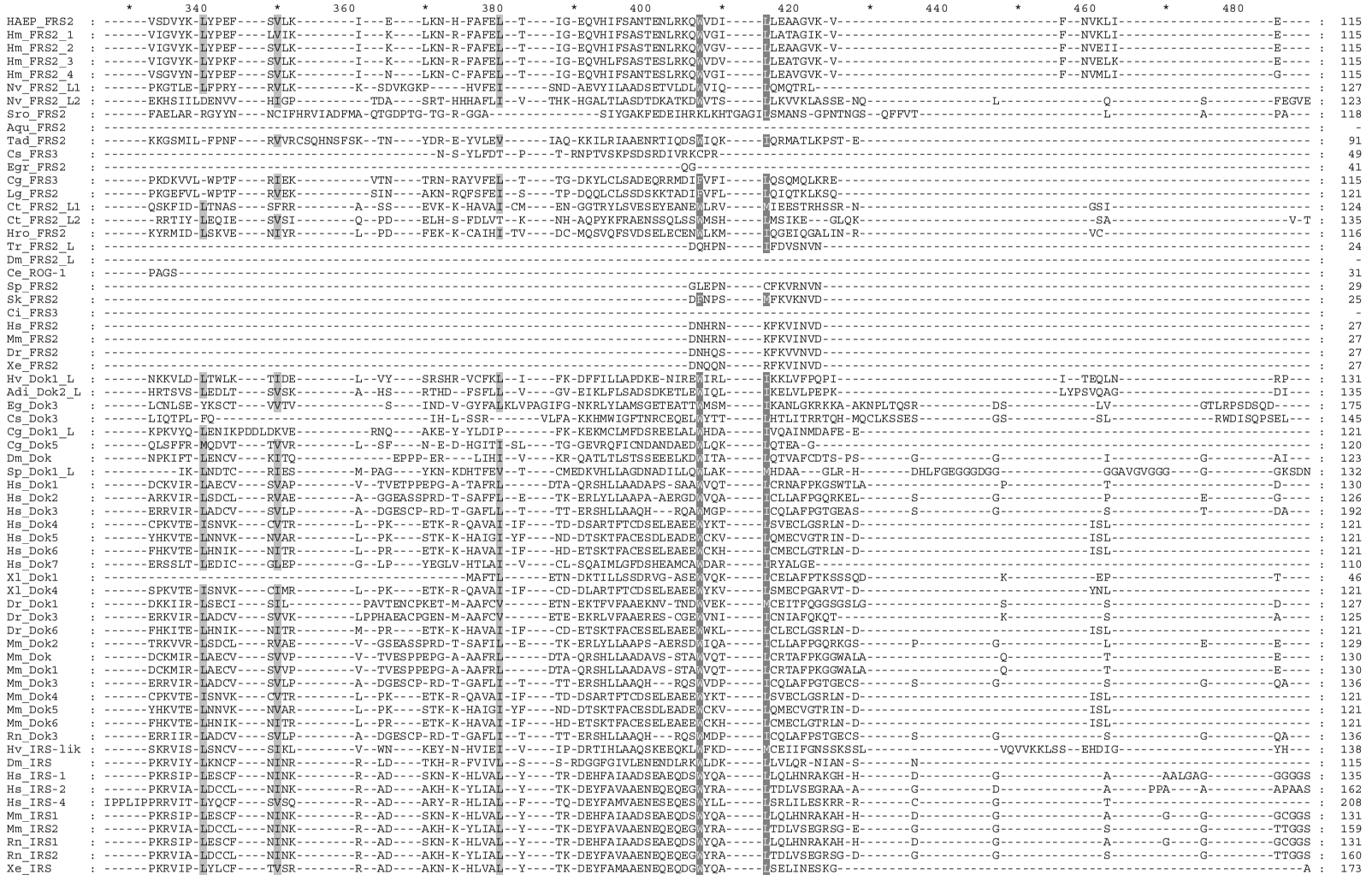


Fig. S12: Alignment of FRS2, PH-FRS2, IRS and Dok proteins. The blue bar indicates localization of the PTB domain. Purple color bar indicates PH domain.

PH- Domain


* 180 * 200 * 220 * 240 * 260 * 280 * 300 * 320
HAEP_FRS2 : 50
Hm_FRS2_1 : 50
Hm_FRS2_2 : 50
Hm_FRS2_3 : 50
Hm_FRS2_4 : 50
Nv_FRS2_L1 : 69
Nv_FRS2_L2 : 49
Sro_FRS2 : 37
Aqu_FRS2 : 9
Tad_FRS2 : 21
Cs_FRS3 : 20
Egr_FRS2 : 39
Cg_FRS3 : 54
Lg_FRS2 : 60
Ct_FRS2_L1 : 58
Ct_FRS2_L2 : 72
Hro_FRS2 : 51
Tr_FRS2_L : -
Dm_FRS2_L : 18
Ce_ROG-1 : 27
Sp_FRS2 : -
Sk_FRS2 : -
Ci_FRS3 : 14
Hs_FRS2 : -
Mm_FRS2 : -
Dr_FRS2 : -
Xe_FRS2 : -
Hv_Dok1_L : 63
Adi_Dok2_L : 65
Eg_Dok3 : 87
Cs_Dok3 : 73
Cg_Dok1_L : 56
Cg_Dok5 : 64
Dm_Dok : 57
Sp_Dok1_L : 47
Hs_Dok1 : 60
Hs_Dok2 : 55
Hs_Dok3 : 123
Hs_Dok4 : 55
Hs_Dok5 : 55
Hs_Dok6 : 55
Hs_Dok7 : 52
Xl_Dok1 : -
Xl_Dok4 : 55
Dr_Dok1 : 57
Dr_Dok3 : 57
Dr_Dok6 : 55
Mm_Dok2 : 58
Mm_Dok : 60
Mm_Dok1 : 60
Mm_Dok3 : 67
Mm_Dok4 : 55
Mm_Dok5 : 55
Mm_Dok6 : 55
Rn_Dok3 : 67
Hv_IRS-lik : 58
Dm_IRS : 53
Hs_IRS-1 : 59
Hs_IRS-2 : 88
Hs_IRS-4 : 137
Mm_IRS1 : 59
Mm_IRS2 : 88
Rn_IRS1 : 59
Rn_IRS2 : 89
Xe_IRS : 112

PH- Domain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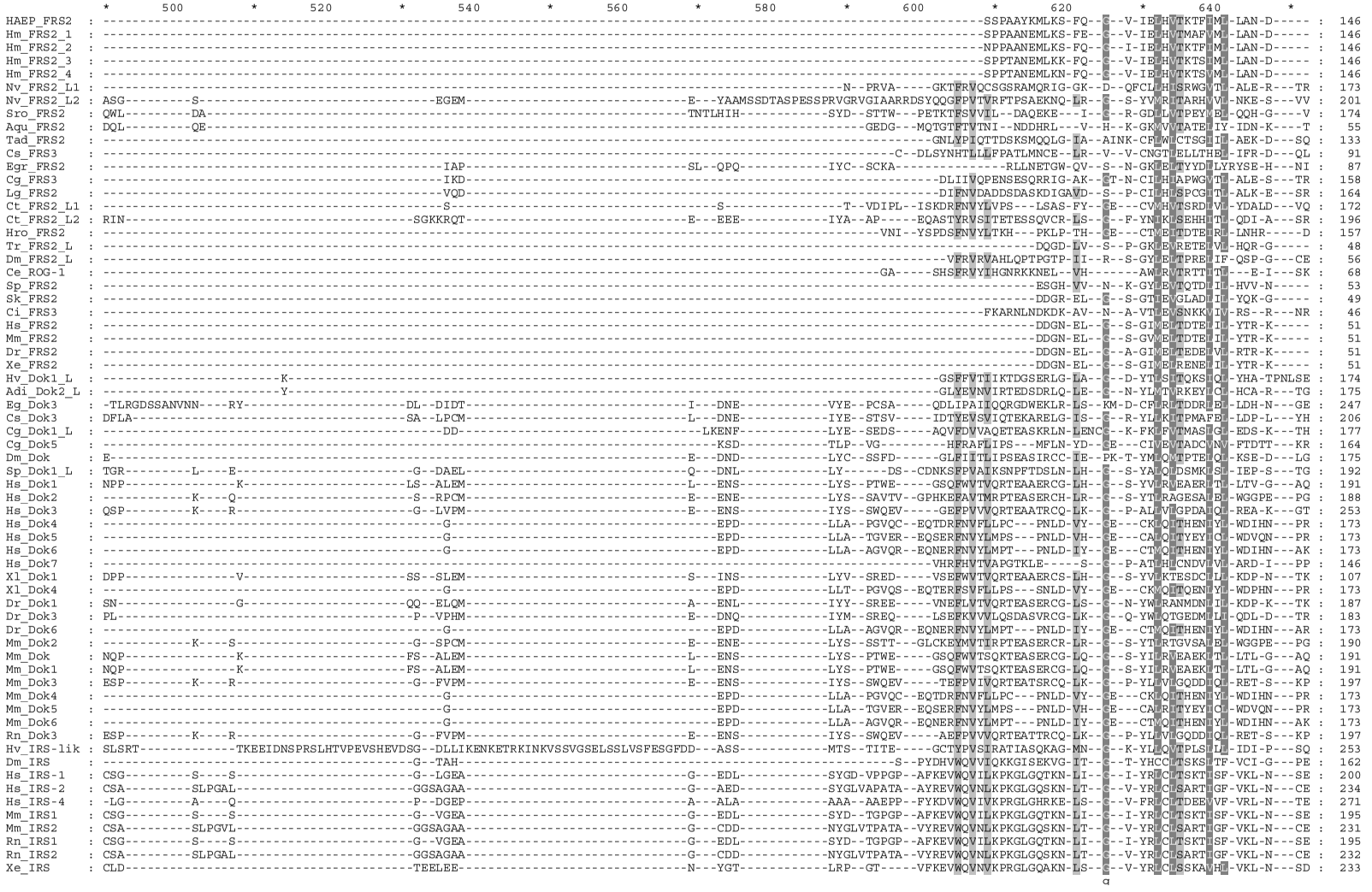
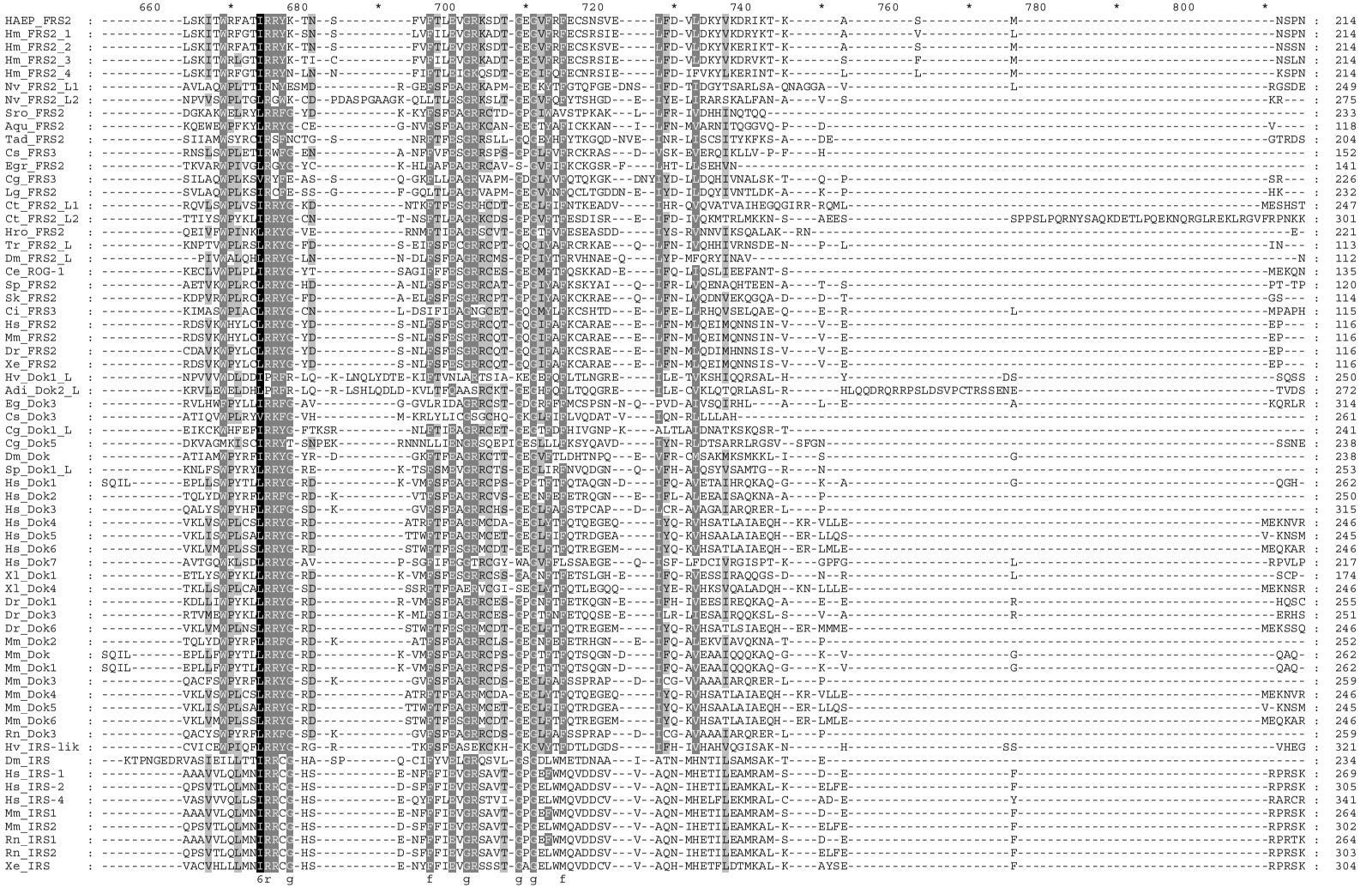
Hm_FRS2_2
Hm_FRS2_3
Hm_FRS2_4
Nv_FRS2_L1
Nv_FRS2_L2
Sro_FRS2
Aqu_FRS2
Tad_FRS2
Cs_FRS3
Egr_FRS2
Cg_FRS3
Lg_FRS2
Ct_FRS2_L1
Ct_FRS2_L2
Hro_FRS2
Tr_FRS2_L
Dm_FRS2_L
Ce_ROG-1
Sp_FRS2
Sk_FRS2
Ci_FRS3
Hs_FRS2
Mm_FRS2
Dr_FRS2
Xe_FRS2
Hv_Dok1_L
Adi_Dok2_L
Eg_Dok3
Cs_Dok3
Cg_Dok1_L
Cg_Dok5
Dm_Dok
Sp_Dok1_L
Hs_Dok1
Hs_Dok2
Hs_Dok3
Hs_Dok4
Hs_Dok5
Hs_Dok6
Hs_Dok7
Xl_Dok1
Xl_Dok4
Dr_Dok1
Dr_Dok3
Dr_Dok6
Mm_Dok2
Mm_Dok
Mm_Dok1
Mm_Dok3
Mm_Dok4
Mm_Dok5
Mm_Dok6
Rn_Dok3
Hv_IRS-like
Dm_IRS
Hs_IRS-1
Hs_IRS-2
Hs_IRS-4
Mm_IRS1
Mm_IRS2
Rn_IRS1
Rn_IRS2
Xe_IRS

PTB- Domain



PTB- Domain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700
720
740
760
780
800
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Hm_FRS2_2
Hm_FRS2_3
Hm_FRS2_4
Nv_FRS2_L1
Nv_FRS2_L2
Sro_FRS2
Aqu_FRS2
Tad_FRS2
Cs_FRS3
Egr_FRS2
Cg_FRS3
Lg_FRS2
Ct_FRS2_L1
Ct_FRS2_L2
Hro_FRS2
Tr_FRS2_L
Dm_FRS2_L
Ce_ROG-1
Sp_FRS2
Sk_FRS2
Ci_FRS3
Hs_FRS2
Mm_FRS2
Dr_FRS2
Xe_FRS2
Hv_Dok1_L
Adi_Dok2_L
Eg_Dok3
Cs_Dok3
Cg_Dok1_L
Cg_Dok5
Dm_Dok
Sp_Dok1_L
Hs_Dok1
Hs_Dok2
Hs_Dok3
Hs_Dok4
Hs_Dok5
Hs_Dok6
Hs_Dok7
Xl_Dok1
Xl_Dok4
Dr_Dok1
Dr_Dok3
Dr_Dok6
Mm_Dok2
Mm_Dok
Mm_Dok1
Mm_Dok3
Mm_Dok4
Mm_Dok5
Mm_Dok6
Rn_Dok3
Hv_IRS-lik
Dm_IRS
Hs_IRS-1
Hs_IRS-2
Hs_IRS-4
Mm_IRS1
Mm_IRS2
Rn_IRS1
Rn_IRS2
Xe_IRS
6r g
f g g g f

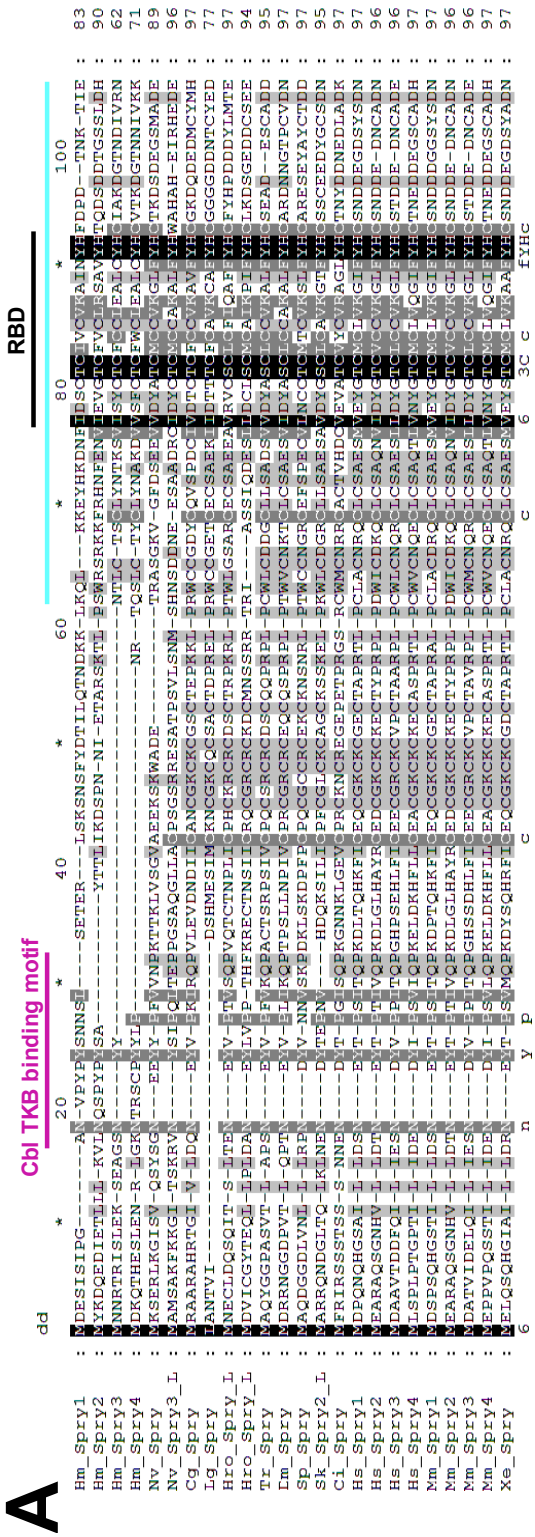
Phylum	Species	Name of the Grb2	Accession numbers	Full length	Name of the Shp2	Accession numbers	Full length	Database for Grb2 and Shp2
Cnidaria	*Hydra vulgaris*	Hv_Grb2_L	XP_002166949.1	Y	Hv_PTPN11_L	XP_002169409.2	Y	GenBank
					Hv_Csw_L	XP_002163154.2	Y	GenBank
	Nematostella vectensis	Nv_Grb2	EDO38076.1	Y	Nv_PTPN11_L	XP_001623905.1		GenBank
Choanoflagellate	*Salpingoeca rosetta*	Sro_Grb2	XP_004995040.1	Y				GenBank
Porifera	*Amphimedon queenslandica*	Aqu_Grb2	XP_003382848.1	Y	Aqu_PTPN11	XP_003388708.1	Y	GenBank
Placozoa	*Trichoplax adhaerens*				Tad_PTPN11_L	XP_002118643.1		GenBank
Platyhelminthes	*Clonorchis sinensis*	Cs_Grb2	GAA48411.1	Y	Cs_PTPN11	GAA56583.1	Y	GenBank
	Echinococcus granulosus							GenBank
Mollusca	*Crassostrea gigas*	Cg_Grb2	EKC35648.1	Y				GenBank
	Lottia gigantea				Lg_PTPN11_L1	ESO94440.1	Y	GenBank
					Lg_PTPN11_L2	ESP03520.1	Y	GenBank
Annelida	*Capitella teleta*	Ct_Grb2	ELU15212.1	Y				GenBank
								GenBank
	Helobdella robusta	Hro_Grb2	ESO09427.1	no				GenBank
Insecta	*Tribolium castaneum*	Tr_Grb2_L	XP_969998.1	Y				GenBank
	Drosophila melanogaster	Dm_Drk	NP_476858.1	Y	Dm_CSW	NP_477131.1	Y	GenBank
	Anopheles gambiae				Ag_PTPN11	ETN60871.1	Y	GenBank
Nematoda	*Caenorhabditis elegans*	Ce_SEM-5	CCD63850.1	Y	Ce_PTP	AAC21678.1	Y	GenBank
Echinodermata	*Strongylocentrotus purpuratus*	Sp_Grb2	XP_001193089.2	Y				GenBank
Hemichordata	*Saccoglossus kowalevskii*	Sk_Grb2	XP_002734614.1	Y				GenBank
Urochordata	*Ciona intestinalis*	Ci_Grb2_L	XP_002131475.1	Y				GenBank
Vertebrata	*Homo sapiens*	Hs_Grb2	CAG46740.1	Y	Hs_PTPN11	NP_002825.3	Y	GenBank
	Mus musculus							GenBank
	Danio rerio	Dr_Grb2	NP_998200.1	Y	Dr_PTPN11	NP_956140.1	Y	GenBank
	Xenopus laevis	Xe_Grb2	AAH74118.1	Y				GenBank

Fig. S13: Names and database accession numbers of all used Grb2 and Shp2 sequences

Phylum	Species	Name of the Sprouty	Accession numbers	Database	Full length
Cnidaria	*Hydra vulgaris*	Hv_Spry2	XP_002155300.2	GenBank	Yes
	Hydra magnipapillata	Hm_Spry1	Hma2.221587	Metazome	Yes
		Hm_Spry2	Hma2.220479	Metazome	Yes
		Hm_Spry3	Hma2.218888	Metazome	Yes
		Hm_Spry4	Hma2.221461	Metazome	Yes
	Hydra vulgaris AEP	HAEP_Spry2	HAEP_T-CDS_v02_47062	Compagen	Yes
	Nematostella vectensis	Nv_Spry	ABN70842.1	GenBank	Yes
		Nv_Spry3_L	AFP87465.1	GenBank	Yes
Choanoflagellate	*Salpingoeca rosetta*				
Porifera	*Amphimedon queenslandica*				
Placozoa	*Trichoplax adhaerens*				
Platyhelminthes	*Clonorchis sinensis*				
	Echinococcus granulosus				
Mollusca	*Crassostrea gigas*	Cg_Spry	EKC37112.1	GenBank	Yes
	Lottia gigantea	Lg_Spry	ESO83378.1	GenBank	No
	Aplysia californica	Ac_Spry	XP_005102499.1	GenBank	Yes
Annelida	*Capitella teleta*	Ct_Spry	ELT89510.1	GenBank	No
	Helobdella robusta	Hro_Spry_L1	ESN92576.1	GenBank	Yes
		Hro_Spry_L2	ESN99557.1	GenBank	Yes
Insecta	*Tribolium castaneum*	Tr_Spry	EFA02831.1	GenBank	Yes
	Drosophila melanogaster	Dm_Spry	AAC04257.1	GenBank	Yes
	Culex quinquefasciatus	Cq_Spry	EDS33107.1	GenBank	Yes
	Aedes aegypti	Aea_Spry	XP_001648910.1	GenBank	Yes
Nematoda	*Ascaris suum*	Asu_Spry	ERG79241.1	GenBank	Yes
Echinodermata	*Strongylocentrotus purpuratus*	Sp_Spry	XP_001176098.2	GenBank	Yes
	Paracentrotus lividus	Pl_Spry	ABO65254.1	GenBank	Yes
Hemichordata	*Saccoglossus kowalevskii*	Sk_Spry2_L	XP_002739802.1	GenBank	Yes
Urochordata	*Ciona intestinalis*	Ci_Spry	XP_002132157.1	GenBank	Yes
	Oikopleura dioica	Odi_Spry	CBY13665.1	GenBank	Yes
Vertebrata	*Homo sapiens*	Hs_Spry1	AAT06102.1	GenBank	Yes
		Hs_Spry2	AAH04205.1	GenBank	Yes
		Hs_Spry3	NP_005831.1	GenBank	Yes
		Hs_Spry4	AAI25097.1	GenBank	Yes
	Mus musculus	Mm_Spry1	NP_036026.1	GenBank	Yes
		Mm_Spry2	AAD34167.1	GenBank	Yes
		Mm_Spry3	NP_001025464.1	GenBank	Yes
		Mm_Spry4	AAD56007.1	GenBank	Yes
	Danio rerio	Dr_Spry2	AAQ22393.1	GenBank	Yes
	Xenopus laevis	Xe_Spry	NP_001153153.1	GenBank	Yes
	Branchiostoma lanceolatum	Bla_Spry	ADU32856.1	GenBank	5' end missing

Fig. S14: Names and database accession numbers of Sprouty sequences

Fig. S16: Alignments of Sprouty proteins used to calculate the phylogenetic trees and Conserved domain figure. A) Only conserved domains alignment, (B) Whole sequence alignment. The violet bar shows sequence of Cbl TKB binding domain, black bar indicates localization of the RBD domain and blue bar indicate Spry domain.



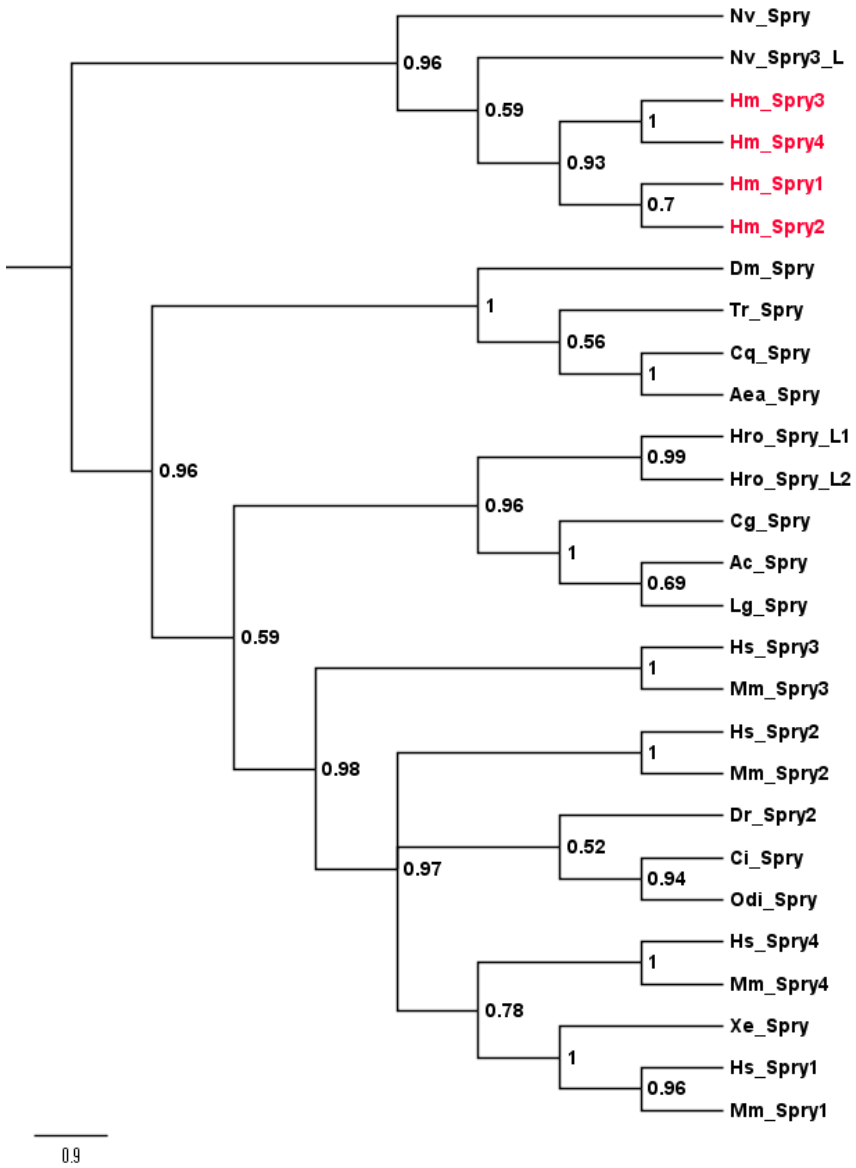


Fig. S18: Phylogenetic tree of conserved domains in Sprouty proteins. Sprouty protein conserved domain sequences (RBD+ Spry domain) from Hm_Spry, *Hydra magnipapillata*7 Nv_Spry, *Nematostella vectensis3* Dm_Spry, *Drosophila melanogaster3* Tr_Spry, *Tribolium castaneum3* Cq_Spry, Culex quinquefasciatus*3* Aea_Spry, *Aedes aegypti3* Cg_Spry, *Crassostrea gigas3* Lg_Spry, *Lottia gigantea3* Ac_Spry, *Aplysia californica3* Hro_Spry_L, *Helobdella robusta* Spry like*3* Ci_Spry, *Ciona intestinalis3* Odi_Spry, *Oikopleura dioica3* Hs_Spry, *Homo sapiens3* Mm_Spry, *Mus musculus3* Dr_Spry2, *Danio rerio3* Xe_Spry, *Xenopus laevis* were used for phylogeny inference. Tree was rooted with Cnidaria family.

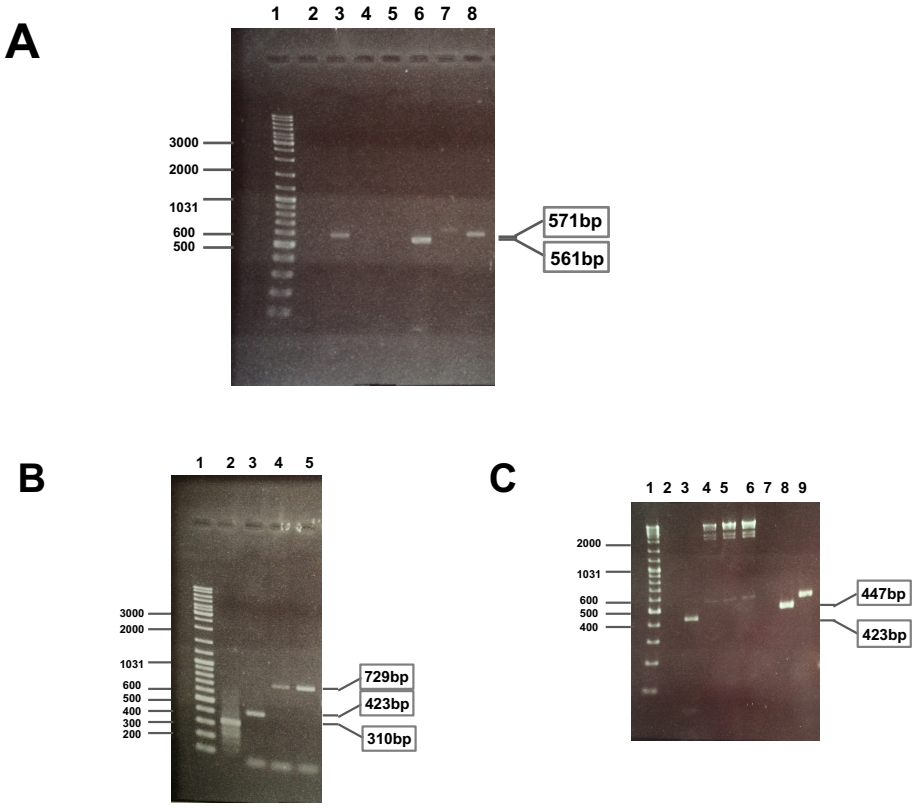


Fig. S19. Gel image of *Hydra* AEP cDNAs amplified by PCR (A)*Dof* and *FRS2* cDNA, (B) *Spry1, Spry2* and *Spry4* cDNA (C) *Spry2* and *Spry3* cDNA.

spry 3

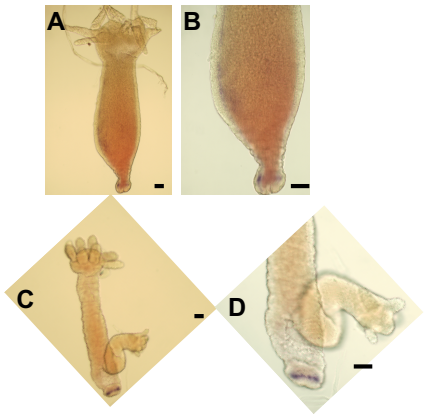


Fig. S21 Expression pattern of *spry3* mRNA in budding *Hydra vulgaris AEP* polyps. (A and C) are overviews and (B and D) are their close ups respectively. Scale bar = 100 µm.

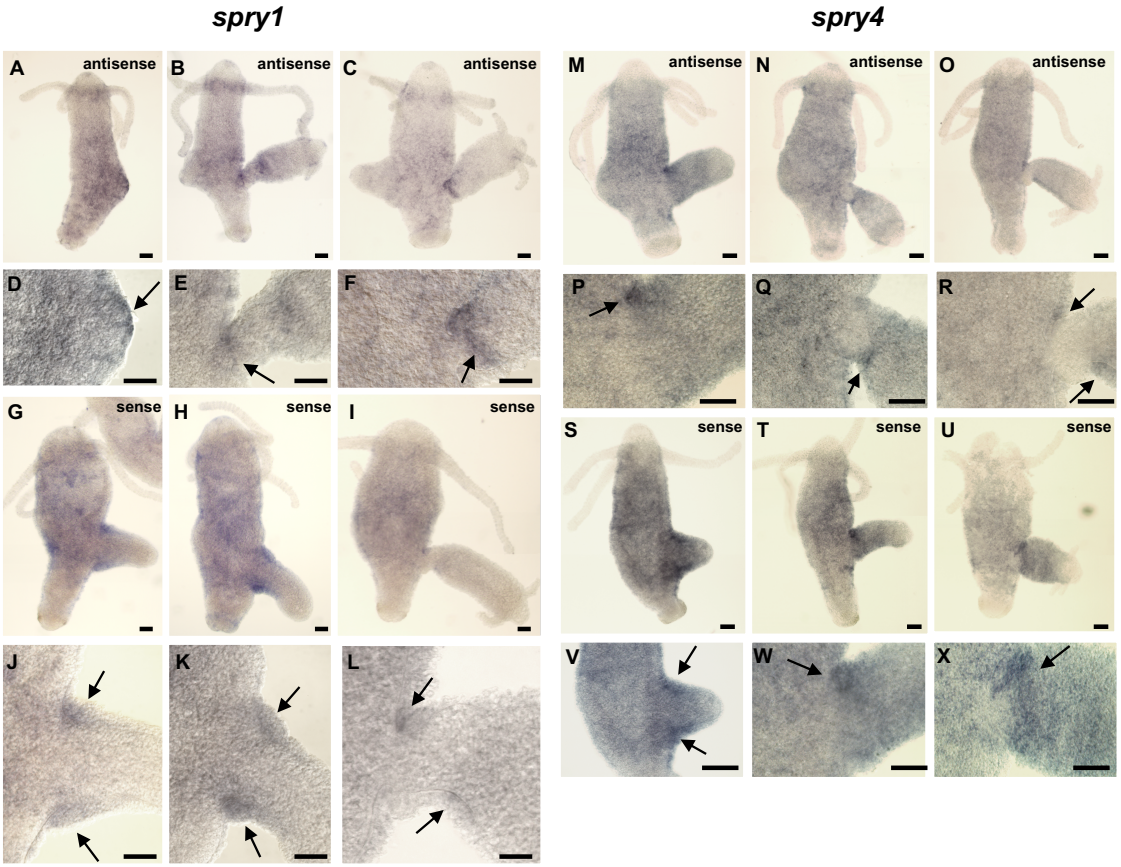


Fig. S20 Expression pattern of *spry1* and *spry4* mRNA in budding *Hydra vulgaris AEP* polyps. (AJF) *spry1* and (MJR) *spry4* antisense mRNA expression. (GJL) *spry1* and (SJX) *spry4* sense mRNA expression. (AJC, GJL, MJ O and SJU) are overviews and (DJF, JJL, PJR and VJX) are their close ups respectively. Scale bar = 100 µm.

Fig. S24: Anti-Spry2 Antibody information:

Anti-Spry2 antibody 1 against peptide: Hm_Spry2 Anti-VIE **(286-303)**

VIEVGTCFVCLRSAVYHT

Anti-Spry2 antibody 2 against peptide: Hm_Spry2 Anti-RSA **(297-316)**

RSAVYHTQDSDDTGSSLDHP

>Hm_Spry2

MYKDQEDIEKDELMWVKAGGPGEKPTIIRQVCSDSSSCSSRENSGESSNGVKQTLLLSPK
VGLTSRVPSNDSMHNFGHLGRTSSEDSRVIVLNVSQSPYPYSIDKEKVKRLKISQGIYAA
SKDEKGSTINNFKTSSFSNNTDDSQVSVCDLILKSKSQPVDFLPRFSPMTAIKRSSSDDS
PQKSSSLNHGYPKTKSKEGYTTLIKDSPNPNISETARSKTLPSSWRPAYSKDHQKDFSKF
SKSIALEQVVSFQDELYNKSSEITTSQVKLKRKKSRKKFRHNFENVIEVGTCFVCLRSAV
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