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Extending the family table: insights into the FGF superfamily from beyond vertebrates

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Abstract

Since the discovery of Fibroblast Growth Factors much focus has been placed on elucidating the roles for each vertebrate FGF ligand, receptor, and regulating molecules in the context of vertebrate development, human disorders and cancer. Studies in human, mouse, *Xenopus*, chick, and zebrafish have gone a long way to help us understand [AS1]which FGFs are involved in which processes. However, in recent years, as more genomes are sequenced, more information is becoming available from many non-vertebrate models and a more complete picture of the FGF superfamily as a whole is emerging. In some cases less redundancy in the FGF signaling system in invertebrate models may allow for more mechanistic insights. Studies in cnidaria have highlighted how ancient FGF signaling is, and helped provide insight into the evolution of the FGF gene family. Work in *C. elegans* has shown that different splice forms can be used for functional specificity in invertebrate FGF signaling. Comparing FGFs from *Ciona* to those in vertebrates and FGFs from *Tribolium* to *Drosophila* reveals some important clues as to the process of gene loss, duplication and subfunctionalization of FGFs throughout evolution. Finally, comparing all members of the FGF ligand superfamily reveals variability in many properties, which may point to a feature of FGFs as being highly adaptable with regards to protein structure and mechanism. Further studies on FGF signaling outside of vertebrates is likely to complement work in vertebrates by contributing many insights to the FGF field as a whole and providing unexpected information that could be used for medical applications.

Introduction

Cell signaling by Fibroblast Growth Factors (FGF) is essential to the development and maintenance of animals. From their discovery in the early 1970s to today, researchers continue to detail the contributions of FGF signaling to developmental and adult metabolic processes. It has become clear that FGF signaling is not limited to a few uses, but has many functions both in developing embryos and the adult. As more genomes are sequenced and more FGF superfamily members are described, the amount of structural and functional variety within the family is becoming apparent and FGF signaling appears to be highly adaptable, helping to make possible the great variety of life forms. In this review we will first highlight the history of FGF research, the structure of the FGF signaling complex, the

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downstream pathways employed and major functional findings from vertebrate FGF research. Then, we will discuss findings from non-vertebrate models in the context of emerging themes in FGF superfamily research.

Historical perspective

A prelude to the discovery of FGFs was the finding in 1939 that bovine brain extracts could cause proliferation of fibroblast cell lines in vitro (Hoffman, 1940; Mohammadi et al., 2005; Trowell OA, 1939). Biochemical characterization of this mitogenic activity did not begin for another 34 years, when a factor in pituitary extracts was found to stimulate growth of 3T3 mouse fibroblast cells and was characterized as being thermolabile, sensitive to proteases and enhanced by hydrocortisone (Armelin, 1973). Gospodarowicz (1974) purified the mitogenic factor from pituitary extracts and found it was also present at higher concentrations in brain extracts. He termed this molecule Fibroblast Growth Factor and showed that with hydrocortisone FGF could stimulate DNA synthesis as effectively as crude serum (Gospodarowicz, 1974). Another important finding from these early studies was the incredible potency of FGF: the minimal effective dose was only 0.1 ng ml^{-1}. It was also found that FGF could induce proliferation of diploid human foreskin fibroblasts and mouse fibroblast cells, showing that FGF lacks species specificity (Gospodarowicz and Moran, 1975). FGF activity was found to be due to a 15 kD molecule and was called basic FGF (bFGF) because of its high isoelectric point (pI) (Gospodarowicz, 1975; Gospodarowicz, 1978). Another molecule with FGF activity was also isolated from brain extracts and was called acidic FGF (aFGF) because of its lower pI (Maciag et al., 1979).

A number of other mitogenic proteins were subsequently found to be chemically identical to either aFGF or bFGF (Burgess and Maciag, 1989; Burgess et al., 1986; Lemmon et al., 1982; Libermann et al., 1987; Mohammadi et al., 2005). More members of the FGF family were found using several approaches and a numbering-scheme was established in which aFGF and bFGF were renamed as FGF1 and FGF2, respectively. FGF3 (INT-2) (Dickson et al., 1984), FGF4 (K-FGF/HST) (Delli Bovi and Basilico, 1987; Sakamoto et al., 1986), and FGF5 (Zhan et al., 1988) were all discovered as oncogenes. FGF6 was identified based on the similarity of its sequence to FGF4 (Marics et al., 1989). FGF7 was discovered with classical protein purification from fibroblasts and this study showed for the first time that FGFs are necessary for tissue homeostasis by enabling communication between mesenchymal and epithelial tissues (Rubin et al., 1989). FGF8 was isolated as an androgen-induced growth factor (Tanaka et al., 1992). FGF9 was found because of its ability to stimulate the growth of glia cells (Miyamoto et al., 1993).

Between 1996 and 2003, other FGFs were found through a combination of bioinformatic tools and homology-based PCR: FGF10 (Lu et al., 1999), FGF16 (Miyake et al., 1998), FGF17 (Xu et al., 1999), FGF18 (Ohbayashi et al., 1998), FGF19 (Nishimura et al., 1999), FGF20 (Kirikoshi et al., 2000), FGF22 (Nakatake et al., 2001), FGF23 (Yamashita et al., 2000), FGF24 (Draper et al., 2003). FGF11-FGF14 make up a subfamily of Fibroblast Homologous Factors (FHFs) that are not secreted and do not bind to FGF receptors (FGFR) 1–4 (Coulier et al., 1997; Smallwood et al., 1996). FHFs can also bind to heparin with high affinity like the canonical FGFs, yet despite striking structural similarity, FHFs have

diverged toward interaction with a separate set of target proteins and do not share functional homology with FGFs (Olsen et al., 2003).

Today, the FGF family represents one of the largest signaling families in vertebrates, with 24 known ligands in total, although not every member is present in every vertebrate species. The first FGF receptor (FGFR) was identified in the mid-1980's (Lee et al., 1989; Olwin and Hauschka, 1986), and since then 4 FGFRs have been found in vertebrates (Coumoul and Deng, 2003).

Structure of FGF ligands, receptors, and signaling complex

FGF ligands share a homologous core domain consisting of 120–130 amino acids ordered into 12 antiparallel β-strands (β1-β12) that are arranged into three sets of four-stranded β-sheets that fold to form a β-trefoil structure (Mohammadi et al., 2005). Additionally, they have variable length N- and C-terminal tails, which largely account for the specific biology of different FGF family members. Most FGFs have traditional signal peptides and are secreted as soluble signaling molecules. Vertebrate FGFs are also known to bind to heparan sulfate glycosaminoglycans (HSGAG) through the HSGAG binding site (HBS), located in the FGF core within the β1-β2 loop and the region between β10-β12. The elements of the HBS form a contiguous, positively charged surface.

FGF ligands bind to the FGFR family of tyrosine kinase receptors in an heparan sulfate proteoglycan(HSPG)-dependent manner. In vertebrates there are 4 FGFRs (FGFR1-FGFR4) which bind to the 24 ligands with varying degrees of promiscuity. The structure of the FGFR consists of three extracellular immunoglobulin domains (D1-D3), a transmembrane domain, and an intracellular tyrosine kinase domain. A unique feature of FGFR is the presence of an acidic, serine-rich sequence in the linker between D1 and D2, which is known as the acid box. The FGF ligands bind to the D2-D3 region of the FGFR ectodomain. The D1 and acid box are thought to play a role in receptor autoinhibition (Mohammadi et al., 2005).

A functional FGF-FGFR signaling unit consists of two 1:1:1 FGF-FGFR-HSGAG complexes that are bound together into a dimer. The ligand of each complex binds to both receptors to allow interaction with each other through a region in the D2 domain. The HSGAG incorporates into the dimer through a "basic canyon" and contributes to dimerization by binding both the ligands and the receptors (Beenken and Mohammadi, 2009). Additionally, HSGAGs stabilize FGFs against degradation, act as a storage reservoir, and can affect the radius of ligand diffusion (Häcker et al., 2005). Dimerization of FGFR allows the cytoplasmic kinase domains to become activated (Mohammadi et al., 1996).

Signaling transduction pathways utilized

Several reviews have been written detailing the research on downstream signaling pathways used by FGF signaling (Böttcher and Niehrs, 2005; Eswarakumar et al., 2005; Thisse and Thisse, 2005), and therefore we will only review briefly here this aspect of FGF signaling. FGFR-stimulation leads to tyrosine phosphorylation of Shp2 resulting in complex formation of Grb2 and its associated nucleotide exchange factor son-of-sevenless (Sos) and Grb2/Sos activate the Ras GTPase, which then activates the mitogen-activated protein kinase (MAPK)

pathway. The final protein in the MAPK pathway is extracellular signal-regulated kinase (ERK) and it enters the nucleus to activate transcription factors (*c-myc*, AP1 and Ets-family members) that will affect FGF target genes.

The MAPK pathway is not the only pathway used by FGF signaling. Mutational analysis of tyrosine766 has shown that the phosphorylation of this tyrosine residue is essential for complex formation with and tyrosine phosphorylation of phospholipase C gamma (PLCγ) (Eswarakumar et al., 2005). PLCγ activation results in the hydrolysis of phosphatidylinositol-4,5-diphosphate (PIP2) to inositol-1,4,5-triphosphate (IP3) and the generation of two second messengers: IP3 and diacylglycerol (DAG). Recruitment to the membrane of PLCγ is mediated by binding of the Pleckstrin homology domain of PLCγ to IP3 molecules. IP3 causes a release of calcium within the cell, which stimulates GEFs that activate the Rap1 GTPase. Rap1 can assist in the maturation of intercellular junctions and mediate adhesion through the recruitment of cadherins and integrins to the plasma membrane. Signaling through the FGFR can thus result in multiple responses: cellular differentiation through Ras GTPase and cell adhesion/migration through PLCγ/Rap1 (Raaijmakers and Bos, 2009).

The PI3 Kinase/Akt pathway can be activated in three ways: (1) Gab can bind to FRS2 via Grb2, (2) the PI3 subunit p85 can bind to a phosphorylated tyrosine residue of the FGFR, and (3) activated Ras can induce membrane localization and activation of the p110 catalytic subunit of P13 kinase (Böttcher and Niehrs, 2005).

The different downstream signal transduction pathways used by FGF signaling can lead to specific cellular response in a cell-type dependent manner (Dailey et al., 2005). For instance, the ERK kinases are generally thought to be responsible for the mitogenic response of cells to FGF, while alternate MAPKs, p38 and JNK MAP kinase are usually associated with inflammatory or stress-response.

Functional information from vertebrate studies

The cumulative data [DM8]from studies on FGF signaling in vertebrate models is difficult to summarize in brief. Reviews on FGF functions in vertebrates have been written at regular intervals and include information on FGFs involved in developmental processes, adult maintenance, disorders and cancer (Beenken and Mohammadi, 2009; Ornitz and Itoh, 2001; Thisse and Thisse, 2005). Among other functions, FGFs are key regulators of development, including: mesoderm induction, gastrulation, limb development, midbrain-hindbrain patterning, and bone formation. In the mouse, FGF4 and FGF8 are required for proper migration of epiblast cells through the primitive streak. In the absence of both FGF4 and FGF8, epiblast cells move into the streak and undergo an epithelial-to-mesenchymal transition, but then most cells fail to move away from the streak (Sun et al., 1999; Thisse and Thisse, 2005). Currently it is thought FGF4 is thought to act as an attractant and FGF8 as a repellent to cells in the streak. [DM9]FGF induction of mesoderm has also been studied in *Xenopus laevis*, where FGF2 was first shown to have mesoderm inducing activity equivalent to the ventrovegetal signal (Slack et al., 1987). More recently, the specific roles of different spliceforms of FGF8, FGF8a and FGF8b, have been found to have different activities in the early specification of mesodermal and neural tissue in the frog. FGF8b is a

potent mesoderm inducer in both explants and whole embryos while FGF8a has little effect on the development of mesoderm (Fletcher et al., 2006).

Limb Development—FGFs have been found to play key roles in the process of limb development. Formation of limb buds and their successful outgrowth is dependent upon FGF signaling and a FGF positive-feedback signaling loop between the limb mesenchyme (progress zone) and the overlying ectoderm, termed the apical ectodermal ridge (AER). FGF4, FGF8, FGF9 and FGF17 are all expressed in the AER. Combinatorial FGF mutant studies resulted in the loss of intermediate skeletal structures while the most distal and the most proximal structures remained intact, leading to a the 'two-signal model,' which describes limb mesenchyme initially being influenced by one signal (likely Retinoic Acid) that influence proximal cell fates and subsequently experience FGF signals from the AER establishing the distal domain. The intermediate domain would then form as a result of interactions at the domain boundary. Sonic hedgehog (Shh) is expressed in a posterior domain of the limb bid called the zone of polarizing activity (ZPA) and a positive feedback loop is established between Shh in the ZPA and FGFs in the AER. Shh is required for the induction and maintenance of *Fgf4, 9, 17* and the maintenance of *Fgf8*, and, reciprocally, FGF signaling from the AER is required to maintain *Shh* expression (Duboc and Logan, 2009)

Brain Patterning—Patterning of the midbrain-hindbrain (MHB) anlage depends on an organizer activity located at the MHB junction, also known as the Isthmus. In vertebrates, FGF8 is expressed in the MHB and is a key component of its organizing activity (Crossley et al., 1996). Loss of midbrain and cerebellar tissue results in a mouse with a severe hypomorphic allele of *Fgf8* (Meyers et al., 1998). FGF17 and FGF18 are also expressed in the MHB and the loss of FGF17 in mouse results in the truncation of posterior midbrain and reduced proliferation of the anterior cerebellum (Maruoka et al., 1998). FGF8 is differentially spliced to generate FGF8a and FGF8b isoforms, which are both expressed at the isthmus/MHB (Sato et al., 2001). In the chick, ectopic FGF8a causes expansion of the midbrain whereas misexpression of FGF8b transforms the midbrain into a cerebellum (Sato et al., 2001). Similarly, in the mouse, ectopic FGF8a results in expansion of the midbrain and ectopic expression of *Engrailed2*, whereas ectopic FGF8b leads to exencephaly and a rapid transformation of the midbrain and diencephalon into an anterior rhombomere1[AS11] fate (Liu et al., 1999). FGF8b also maintains two negative feedback loops by inducing the expression of the negative feedback FGF inhibitors Sprouty1 and Sprouty2 and repressing FGFR2 and FGFR3 (Liu et al., 2003). In Zebrafish, FGF8 is also present at the MHB and acts as a morphogen to pattern the midbrain. *Acerebellar* mutants, in which the FGF8 gene contains a premature stop codon, lack a functional MHB and also lacks a cerebellum (Reifers et al., 1998).

Bone Formation—FGF signaling is capable of regulating genes at all steps of osteogenesis. A point mutation in the transmembrane domain of FGFR3 was found to be the etiology of Acondroplasia, the most common genetic form of human dwarfism (Rousseau et al., 1994; Shiang et al., 1994). Missense mutations have since been found in more than 15 human bone disorders, from skeletal dysplasias to short stature. FGF2, FGF9, and FGF18

are all found in osteoblasts. Overexpression of FGF2 in mouse causes abnormal bone formation and loss-of-function of FGF2 leads to inhibition of bone formation (Coffin et al., 1995; Montero et al., 2000). FGF signaling seems to positively regulate cell proliferation and differentiation in osteogenesis. Additionally, FGFs can control apoptosis in osteoblasts when high levels of FGF signaling can reduce apoptosis in immature osteoblasts and increase the total osteoblast population.

Other developmental functions for FGFs in vertebrates have been described as well in many tissues: the nervous system, epidermis, lungs, mammary glands, somite boundaries, ear, kidney, liver, and pancreas (Coleman-Krnacik and Rosen, 1994; Delaune et al., 2005; Kobberup et al., 2010; Sawada et al., 2001; Thisse and Thisse, 2005; Wilkie, 2005).

Insights from non-vertebrate models

An Introduction to non-vertebrate FGFs and FGFRs

FGF signalling has now been described in a number of model systems outside of vertebrates including the echinoderm sea urchin *Strongylcentrotus purpuratus*, the urochordate ascidians *Ciona intestinalis* and *Ciona savigny*, the ecdysozoans *Caenorhabditis elegans*, *Drosophila melanogastor*, and *Tribolium castaneum*, and the anthozoan cnidarian, *Nematostella vectensis*. The relationship of these groups to vetebrates is summarized in Figure 1.

This list will surely expand in the near future, but it is worth surveying the current described members of the FGF family outside of vertebrates (Table 1). In the sea urchin, they have identified one ligand, FGFA, and two receptors, FGFR1 and FGFR2 (Lapraz et al., 2006; McCoon et al., 1996; McCoon et al., 1998; Röttinger et al., 2008). The ligand was called FGFA because the predicted protein showed similarities to both the FGF8 and FGF9 subfamilies and phylogenetic analysis gave ambiguous results. Ciona has 6 FGF ligands and 1 receptor: *Ci*-FGF8/17/18, *Ci*-FGF11/12/13/14, *Ci*-3/7/10/22, *Ci*-FGF4/5/6, *Ci*-FGF9/16/20, *Ci*-FGFL (FGF with large molecular mass), and *Ci*-FGFR (Satou et al., 2002; Shi et al., 2009). In *Drosophila*, there are three FGF ligands: Branchless (Bnl), Thisbe (Ths), and Pyramus (Pyr). Ths and Pyr are most related to the FGF8 subfamily. Additionally, there are two FGFRs: Bnl uses the Breathless FGFR (Btl), and Ths and Pyr signal through the Heartless receptor (Htl). *Tribolium* has 4 FGF ligands and a single FGFR: *Tc*-FGF1a, *Tc*-FGF1b, *Tc*-FGF8, *Tc*-Branchless (*Tc*-Bnl), and *Tc*-FGFR (Beermann and Schröder, 2008). In *C. elegans*, there are two FGF ligands, egl-17 and LET-756, and one FGFR, egl-15. Egl-17 is most similar to the FGF8 subfamily and LET-756 to the FGF9 subfamily. In the anthoszoan cnidarian *Nematostella vectensis*, there are 4 ligands and 2 receptors: *Nv*FGF8A, *Nv*FGF8B, *Nv*FGF1A, *Nv*FGFa2, *Nv*FGFRa, and *Nv*FGFRb (Matus et al., 2007; Rentzsch et al., 2008). A probable FGFR, *kringelchen*, has been identified in the hydrozoan cnidarian *Hydra* (Sudhop et al., 2004). Two FGFRs, *Dj*-FGFR1 and *Dj*-FGFR2 have been found in the platyhelminthes planarian *Dugesua japonica*, rounding out representatives from all the major metazoan phyla (Ogawa et al., 2002).

The role of FGFs in development is an ancient one

FGF signaling is an ancient cell-to-cell communication system as evidenced by its presence in the cnidaria, which split off from its sister group bilateria an estimated 600 million years ago (Rentzsch 2008). *Nematostella vectensis*, a sea anemone, is considered to be a representative of basal cnidarians and to have retained much of the genetic complexity contained in the cnidarian-bilaterian ancestor (Bridge et al., 1995; Bridge et al., 1992; Chourrout et al., 2006; Collins et al., 2006; Medina et al., 2001; Putnam et al., 2007; Ryan et al., 2006; Technau et al., 2005). The two FGFRs identified in *Nematostella*, *NvFGFRa* and *NvFGFRb* are thought to have arisen from a lineage-specific duplication, and therefore, it is thought likely that there was only 1 FGFR in the cnidarian-bilaterian ancestor (Rentzsch et al., 2008). As many as 15 putative transcripts sharing homology to FGF domains were found via bioinformatic analyses in the *Nematostella* genome, but so far only four have been described: NvFGF1A, NvFGFa2, NvFGF8A, NvFGF8B (Matus et al., 2007; Rentzsch et al., 2008).

In bilaterians, FGF ligands and FGF receptors are often expressed in separate germ layers or tissues and signal across epithelial-mesenchymal boundaries. Yet, in diploblastic cnidarians there is no mesoderm for FGFs to signal to/from, and so the ligands and receptors are expressed in the same domain (*NvFGF1A, NvFGFa2, NvFGFRa)*, or in abutting ectoderm/endoderm tissues of the aboral pole (*NvFGF8A, NvFGFRb*).

Morpholino knockdown of NvFGF1A and NvFGFRa showed that they are required for formation of the apical organ (Rentzsch et al., 2008). Apical organs with a ciliated tuft are also present in both protostomes and deuterostomes: in the larvae of sea urchins, hemichordates, and the polychaete *Platynereis*, although the evolutionary relationship of cnidarian, protostomian and deuterostomian apical organs has not yet been determined. Intriguingly, FGFs or FGFRs are expressed in the region of apical organ formation in sea urchin, hemichordates and polychaetes, leading to the possibility of an ancient function in apical organ formation.

A tyrosine kinase receptor with similarity to FGFR, *kringelchen*, has also been identified in the hydrozoan cnidarian *Hydra*, where it was shown to be essential for boundary formation and tissue constriction as a prerequisite for proper bud detachment which is essential for reproduction (Sudhop et al., 2004). It has yet to be shown that this receptor can actually bind FGFs, which have not been described yet for *Hydra*.

Importance of tight regulation in FGF signaling

Evidence from many systems has pointed to the importance for tight regulation of FGF signaling activity, and the loss of such regulation often leads to developmental disorders and disease. A negative regulator of FGF signaling, Sprouty, was originally identified in *Drosophila* for its action during tracheal development (Hacohen et al., 1998). Sprouty is thought to act in a negative-feedback regulatory loop during FGF and EGF signaling (Casci et al., 1999; Kramer et al., 1999; Sivak et al., 2005). There are four mammalian Sprouty proteins and three related Spreds (Sprouty-related EVHI domain proteins). Sproutys have been found in synexpression groups with FGFs and FGFRs in other nonvertebrate systems.

Nematostella Sprouty, *Nv-Sprouty*, is expressed in the same domain as NvFGF8A, NvFGF8B and NvFGFRa in the apical pole (Matus et al., 2007). The expression of the sea urchin *sprouty* largely follows that of *fgfA* from the late mesenchyme blastula/early gastrula to pluteus stages in bilateral regions of the ectoderm, in the PMC clusters, and at the tip of the growing arms of the larva (Röttinger et al., 2008). Two other probable FGF target genes, *pea3* (Polyoma enchancer activator 3), an Ets domain transcription factor, and paired transcription factor *pax2/5/8*, were also expressed along with *fgfA* and *sprouty* (Röttinger et al., 2008). Sprouty proteins can have a therapeutic effect on some mouse models of disease by enhancing angiogenesis and neovascularization (formation of new blood vessels from preexisting ones) (Taniguchi et al., 2009). Many of the studies in vertebrates relied on double mouse knockouts for combinations of different Sproutys and Spreds. Studies on Sprouty proteins in nonvertebrate models may aid in the further characterization of the mechanism of regulation without the concern of redundancy.

Regulation has also been found to come from certain FGF ligands themselves when co-expressed in the same domain as the functioning ligand. In *Nematostella*, NvFGFa2 negatively regulates FGF signaling at the apical pole, as a morpholino against NvFGFa2 causes the expansion of the apical tuft region along with the expansion of expression of NvFGF1A and NvFGFRa (Rentzsch et al., 2008). This may be related to the function of FGFRL1 molecules (see below, Survey Approach to FGFRL1).

Multiple isoforms of FGFs and FGFRs are generated by splicing

The possible ligand-receptor combinations in vertebrates are numerous and increased by different receptor splice forms. Multiple isoforms are thought to contribute to ligand-receptor specificity and functional specificity. Several examples are also present outside of vertebrates of alternate splice forms of FGFs and FGFRs contributing to functional specificity. *C. elegans* has two ligands LET-756 and EGL-17 and a single receptor, EGL-15 (Birnbaum et al., 2005). EGL-15 is located on the X chromosome and encodes two isoforms, EGL-15(5A) and EGL-15(5B), which result from alternative splicing of exon 5. It has been shown genetically that the different isoforms mediate signaling through two different modules, each using a specific ligand. *Egl-15(5A)* interacts with *egl-17* to mediate sex myoblast chemoattraction and *egl-15(5B)* carries out an essential function required for viability, presumably through signaling by *let-756* (Goodman et al., 2003). Perhaps multiple isoforms are especially important when a single receptor is required to mediate separate functions from two different ligands.

Ciona FGF8/17/18 has two alternative forms of transcripts, that differ in their N-terminal regions (Satou et al., 2002). However, one form is missing the N-terminal region of the FGF domain and whether it is used for signaling and/or regulation is not known.

FGFs have been lost, duplicated and undergo subfunctionalization

Characterizing the complement of FGF ligands in non-vertebrate taxa has provided insight into the evolution of the FGF superfamily across the Metazoa (Popovici et al., 2005). It is clear that in some lineages FGF/FGFR genes have been lost, where in other cases they have been duplicated once or multiple times. Comparisons of FGFs in *Ciona* to vertebrates

reveals that at least two rounds of duplications of most FGF ligands and the FGFR were necessary to account for the multiple subfamily members in vertebrate genomes. It is generally thought that this is consistent with the "2R hypothesis," which maintains that two rounds of whole genome duplication occurred at the base of vertebrate ancestry (Holland et al., 1994; Ohno, 1970).

It has been proposed that the 7 FGF subfamilies present in vertebrates (A–G) plus 1 additional subfamily lost in deuterostomes (H) represent what was once 8 proto-FGF genes in the protostome-deuterostome ancestor (Popovici et al., 2005). *Ciona* has six FGFs, 2 of which were confidently assigned to FGF subfamilies D and F (*Ci*-FGF8/17/18 and *Ci*-11/12/13/14) (Satou et al., 2002). Probable placement in subfamilies B, C, and E was made for an additional 3 FGFs in *Ciona* (*Ci*-FGF3/7/10/22, *Ci*-4/5/6, and *Ci*-9/16/20). The last FGF in *Ciona*, *Ci*-FGFL is characterized by its large predicted molecular mass and could not be assigned to any particular FGF subfamily with confidence. Possible assignments include grouping with other invertebrate FGFs like Branchless in subfamily H, or as a member of subfamily A, B or G (FGF1/FGF2, FGF3/7/10/22 and FGF15/FGF19/FGF21/FGF23, respectively) but its sequence has diverged beyond the similarity required for phylogenetic analysis (Popovici et al., 2005; Satou et al., 2002).

Over time duplicated genes can undergo subfunctionalization to take over different responsibilities. In some cases the combined functions of the two genes equal the function of the original gene, and sometimes the presence of a "backup" gene allows the duplicate or original gene to explore new functional space.

Ciona vs vertebrate FGFs—Many functional studies have been performed on FGFs in *Ciona* and comparisons to studies in vertebrates yield some important similarities (Beh et al., 2007; Bertrand et al., 2003; Davidson et al., 2006; Imai et al., 2002; Kourakis and Smith, 2007; Shi et al., 2009; Yasuo and Hudson, 2007). *Ci*-9/16/20 has been shown to be involved in the induction of notochord, induction of mesenchyme, and heart specification (Davidson et al., 2006; Imai et al., 2002). *Ci*-9/16/20 is expressed adjacent to the heart-producing B7.5 lineage and morpholino knockdown of *Ci*-9/16/20 results in the disruption of heart lineage markers *Mesp, NoTrlc/Hand-like, Tolloid, FoxF* (Davidson et al., 2006; Imai et al., 2006). FGF9 and FGF16 are also known to be involved in heart development in the mouse. Knockout mice for both FGF9 and FGF16 (but not a double mutant) have been generated and have a similar phenotype of reduced number of cardiomyoctes and smaller embryonic heart (Hotta et al., 2008; Lavine et al., 2005). FGF9 and FGF16 are thought to act synergistically to promote the proliferation of embryonic cardiomyocytes. Epicardial and endocardial FGF9/FGF16 signaling through FGFR1/FGFR2 is essential for myocardial proliferation and differentiation (Lavine et al., 2005). In this case it seems that the vertebrate paralogs FGF9 and FGF16 have retained a function in heart development (although possibly not homologous) compared to FGF9/16/20 in *Ciona*. FGF9 and FGF16 seem to be function redundantly at this stage of development with no subfunctionalization apparent.

Ciona FGF3/7/10/22 is expressed in the ventral midline of the neural tube and is important for convergent extension movement in the developing embryo (Shi 2009). In the *Xenopus*

neurula FGF signaling has been implicated in axial elongation as well and possibly a similar mechanism is at play, however the details are still unclear (Sivak et al., 2005).

Ciona FGF8/17/18 is expressed in the nervous system of ascidian embryos and is thought to play a similar role to the patterning of the brain territories that FGF8/FGF17/FGF18 play in vertebrates (see midbrain-hindbrain section of introduction). *Ci*-FGF8/17/18 is expressed in the developing central nervous system (CNS) in a region analogous to the MHB of vertebrate embryos and has led to the hypothesis that a precursor to the organizing activity of FGF8 in the MHB in vertebrates was this region of *Ci*-FGF8/17/18 expression bewteen *Otx* and *Hox* genes in *Ciona* (Ikuta and Saiga, 2007; Imai et al., 2002). Interestingly, 3 other *Ciona* FGFs are also expressed in the developing CNS: *Ci*-9/16/20, *Ci*-3/7/10/22, and *Ci*-FGFL (Imai et al., 2002). Morpholino knockout analysis of *Ci*-FGF8/17/18 has revealed that this ancestor of FGF8/FGF17/FGF18 plays a central role in generating regional patterns of gene expression as morphants have altered expression of *Otx, en, FoxB, Pax2/5/8*, and *Hox 1* (Imai et al., 2009). In vertebrates, FGF17 and FGF18 are also expressed in the mid/hindbrain in a broader domain than FGF8 that includes posterior midbrain (Maruoka et al., 1998). Loss of one copy of *fgf8* in an *fgf17* mutant background results in an exaggerated cerebellum phenotype (Xu et al., 2000). Ectopic FGF8 studies in the chick showed that only ectopic FGF8 leads to the expression of *Engrailed-2*, an early marker of mes/rhombencephalic development, *Wnt1*, and *Fgf8* (Crossley et al., 1996). Ectopic FGF8 can also lead to expression of *Engrailed-1, Pax2* and *Pax5*, and suppression of *Otx2* expression (Liu et al., 1999; Martinez et al., 1999; Shamim et al., 1999; Sheikh and Mason, 1996). It therefore appears that FGF8, FGF17 and FGF18 have already undergone some degree of subfunctionalization in this territory and are not completely redundant.

Drosophila versus Tribolium—Recent analysis of the fully-sequenced genome of the flour beetle, *Tribolium castaneum*, has revealed 4 FGF ligands (*Tc*-FGF1a, *Tc*-FGF1b, *Tc*-FGF8, *Tc*-Bnl) and 1 FGF receptor (*Tc*-FGFR) are present (Beermann and Schröder, 2008). *Tribolium* and *Drosophila* are >300 million years diverged, yet there is some conserved microsynteny between FGF genes in the two species. The gene adjacent to *pyramus* (CG13197, a predicted tyrosine phosphatase) is homologous to the gene upstream of *Tc*-FGF8, *Tc*-00277.

There is only one member of the FGF8 subfamily in *Tribolium*, Tc-FGF8, but two in *Drosophila*, *thisbe* and *pyramus*. The duplication to produce *thisbe* and *pyramus* is thought to have occurred in the arthropod phylum before the radiation of insects because *ths/pyr*-like sequences were found in one study to be represented in both dipterans and hymenopterans (Popovici et al., 2005). However, the presence of only one FGF8 homolog in *Tribolium* supports a different scenario where the duplication occurred in Dipterans. Alternatively, a second FGF8-homolog may have been lost in the *Tribolium* genome. Genes similar to *thisbe* and *pyramus* are present in all other *Drosophila* genomes sequenced so far (unpublished observations), and further investigation of other insect genomes may allow us to point with greater accuracy to the time in which this gene underwent duplication.

Ours and other labs are working on piecing together the overlapping and distinct functions of *pyramus* and *thisbe* to understand how much functional redundancy remains and how far

the process of subfunctionalization has gone in *Drosophila*. Both *pyr* and *ths* function during gastrulation, specification of mesodermal subtypes, migration of caudal visceral mesoderm, and in axonal migration and glial cell wrapping. In the axon there is a clear separation of function for *pyr* and *ths*. Glial-derived *pyr* modulates glial cell numbers and motility whereas neuronal-derived *ths* induces glial differentiation (Franzdóttir et al., 2009). Both ligands were found to influence mesoderm spreading, whereas *pyr* is the dominant player controlling Eve-positive cell specification in the dorsal mesoderm (Kadam et al., 2009; Klingseisen et al., 2009). It therefore seems that the subfunctionalization of *pyr* and *ths* from their insect FGF8-homolog ancestor is underway and *pyr* may either have some derived functions or taken over functions once performed by the single gene.

Studies in *Tribolium* have shown the *pyr/ths* homolog, *Tc*-FGF8, is expressed in largely the same domains as *pyr/ths* during embryogenesis, and so this gene is also likely involved in spreading of the mesoderm, gut development and brain regionalization (Beermann and Schröder, 2008). *Tc*-FGF8 is expressed in the developing brain during mid-segmentation. A stripe of *Tc*-FGF8 expression in each head lobe divides the brain into a larger anterior and a smaller posterior region, in a manner possibly analogous to the MHB in vertebrates. The *Drosophila* embryonic brain is also divided into a tripartite pattern with an anterior *orthodenticle (otd)* and posterior *Hox* domain and an intervening domain. *pyr* and *ths*, however, are not expressed in this middle region, but are expressed in one neuroblast in the anterior compartment in each hemibrain (Urbach 2007). Further functional characterization of *Tribolium* FGFs will undoubtedly provide even more interesting comparisons to *Drosophila* FGFs.

There are two members of the FGF1 subfamily in *Tribolium*, yet there is no member of the FGF1 (A) subfamily in *Drosophila*. This indicates that *Drosophila* has lost the FGF1 subfamily. This is corroborated by the fact that the neighboring genes (*sex-lethal interactor, sin*, and *seven-in-absentia, sina*) to FGF1a and FGF1b in *Tribolium* have conserved gene order in *Drosophila*, but FGF1 is missing in *Drosophila* (Beermann and Schröder, 2008). FGF1 is ubiquitously expressed and is known to play a developmental and maintenance role of neuronal tissue (Beenken and Mohammadi, 2009). Possibly other genes in *Drosophila* have taken over this function.

FGF variability and plasticity

FGFs are most conserved in the “core” FGF domain, however the conservation is often weak, making phylogenetic analysis difficult. Other properties of FGF ligands including secretion signals, homodimerization ability, glycosylation modifications, binding to HSPGs, and other nonconserved domains in N- and C-terminal tails, can vary from molecule to molecule. There is clearly a high level of plasticity in FGF signaling, the reason for which is unknown but likely relates to the complex networks of regulation that these molecules are involved in (Popovici et al., 2005). The 2nd and 3rd extracellular immunoglobulin (Ig) domains of the FGF receptor are involved in binding the FGF ligands. The amino acid sequence constraints imposed on Ig domains are less than for other protein domains, like kinase domains (Popovici et al., 2005). The variability in the amino acid sequence of Ig

domains relates to the high degree of variability in the amino acid composition of FGF ligands (Popovici et al., 2005).

The FGF core domain is thought to be largely responsible for receptor binding. However, the N- and C-terminal tails of FGF molecules are also thought to participate in FGF ligand-receptor specificity. The N- and C-terminal tails can be of variable length. *Drosophila* FGF have extraordinarily long C-terminal domains compared to the average FGF family member, rendering them ~80kD in molecular weight compared to 18–30kD for the average FGF ligand. *Ciona* also has a FGF with a large molecular mass, called *Ci*-FGFL. So far *Ci*-FGFL has not been assigned to a particular FGF subfamily. Despite the evidence for the importance of the sequence at the N- and C- termini, the function of nonconserved domains outside the FGF domain has received little attention in most FGFs. Three notable exceptions are the study of FGF9/FGF20 and FGF23 in vertebrates and LET-756 in *C.elegans*.

The crystal structures of both FGF9 and FGF20 were elucidated and, unlike other FGF ligands, the N- and C-terminal regions were found to be ordered and involved in the formation of a homodimer (two FGF9 ligands or two FGF20 ligands), which obscures the receptor binding site (Kalinina et al., 2009; Plotnikov et al., 2001). The homodimerization and ratio of dimers to monomers appears to autoregulate the ligands receptor binding ability to diffuse through the ECM and bind to HSPGs (Harada et al., 2009; Kalinina et al., 2009).

FGF23 is part of a subgroup of endocrine FGFs. Full length FGF23 is 251 amino acids and is cleaved in the C-terminal tail by subtilisin-like proprotein convertases between amino acids 179 and 180. In humans, failure of this cleavage step results in secretion of additional full-length FGF23, which can cause hypophosphatemia leading to autosomal dominant hypophosphatemic rickets/osteomalacia (Benet-Pagès et al., 2004; Fukumoto, 2005).

The C-terminus of *C. elegans* LET-756 has been shown to contain several nuclear localization signals and the function of them appears to be shuttling LET-756 between several nuclear compartments (Popovici et al., 2006). Additionally, some nuclear localization signals are redundant, highlighting the importance of nuclear localization for LET-756, which has a viability function in *C. elegans*. Subnuclear localization is important for function and LET-756 may be implicated in mRNA splicing machinery and ribosome function (Popovici et al., 2006).

Recently, our lab has also undertaken the task of elucidating the function of the C-terminal domains of Ths and Pyr [AS23]in *Drosophila* (Tulin and Stathopoulos, in review). We found that despite their long length, these domains are not required for activity as truncated constructs removing the C-terminus are functional in an overexpression assay. Additional chimeric constructs revealed that the C-terminus of Ths, but not that of Pyr, may play a role in the rate of ligand diffusion and/or potency. We also provide evidence that *Ths* and *Pyr* are cleaved from their full-length forms into smaller FGFs in cell culture and these cleaved forms are detectable in the embryo as well. In the embryo, cleaved forms of the FGF ligands could be used to support long-range versus short-range functions as might be necessary during the sequential steps of mesoderm migration, specifically in the control of mesoderm tube collapse versus monolayer formation (McMahon et al., 2010).

Use of the survey approach in FGFRL1

The ability to survey genomes from all major metazoan phyla is a powerful tool that allows researchers to understand the degree of conservation of orthologous genes and to investigate questions about whether similar mechanisms are being used. A good example of this approach being used to study FGF signaling is seen in the study of FGFRL1 (fibroblast growth factor like 1). FGFRL1, or FGFR5, is the most recently discovered member of the FGFR family and has an ectodomain with high similarity to conventional FGFRs, but lacks the catalytic tyrosine kinase domain in the intracellular domain (Sleeman et al., 2001; Wiedemann and Trueb, 2000). FGFRL1 mutant mice die immediately after death with a hypoplastic diaphram and also display skeletal alternations, craniofacial dysplasia, heart valve defects, embryonic anemia, and defective kidney development (Baertschi et al., 2007; Catela et al., 2009; Gerber et al., 2009). Initially it was thought that FGFRL1 was limited to vertebrates, but Bertrand and colleagues have shown that there are orthologs in all metazoan phyla and suggest it may represent a conserved regulatory mechanism for attenuating FGF signaling (Bertrand et al., 2009). Some FGFRL1 orthologs have already been identified, such as FGFRL1 in sea urchin, and others remain to be further investigated, like the putative *Drosophila* ortholog CG31431 and the ortholog predicted in the cnidarian *Nematostella*. Subsequent work on FGFRL1 in cell culture and *Xenopus* embryos has revealed that increasing amounts of FGFR1 ectodomain is shed from primary myoblast cells when they begin differentiating into myotubes (Steinberg et al., 2010). FGFRL1 was found to bind several FGF ligands in both its membrane bound soluable state with high affinity. The affinity of FGFRL1 for FGF3 is 1 order of magitude higher than the affinity of FGF3 for its cognate receptor, FGFR2b, consistent with the model that FGFRL1 could act as a decoy receptor to sequester ligand and attenuate signaling through FGFRs. Ectopic expression of FGFRL1 in the *Xenopus* embryo resulted in a similar defect to that of the known phenotype of a dominant-negative form of FGFR1, XFD, and could be rescued by injection of FGFR mRNA.

The mechanism of FGF regulation by FGFRL1 type molecules appears to be widespread. The platyhelminthes planarian *Dugesua japonica*, has a FGFRL molecule called *nou-darake*, has been characterized as also having a similar phenotype as XFD in *Xenopus* embryos (Cebrià et al., 2002).

There are still several unknowns with respect to FGFRL1, including the identity of the protease responsible for shedding the ectodomain, the developmental processes and specific FGF receptors it acts on during normal development, and the biological importance of a polymorphism present in the human population affecting an amino acid involved in cleaving FGFR1 (Steinberg et al., 2010). It will be exciting to see if similar mechanisms of FGF regulation are present in phyla as far as Cnidaria and if work on orthologs in other models can help answer the lingering questions as to the role of FGFRL1 in regulating FGF signaling.

Conclusions and Outstanding Questions

In the context of the FGF superfamily, the mounting number of non-vertebrate FGFs is adding to our knowledge of the evolution of FGF signaling and the variety of mechanisms

available to these growth factors to regulate embryonic development. Important studies from invertebrates have provided models of alternate splicing, subfunctionalization, regulation by Sprouty proteins, and structural plasticity.

FGF signaling is important for human development and human health; therefore, research will undoubtedly continue in all of the discussed areas and will likely provide targets for medical applications. Importantly, FGF signaling is an ancient metazoan cell communication mechanism predating the cnidarian – bilaterian divergence in the pre Cambrian (>600 million years ago), and is utilized by all extant taxa surveyed to date.. [DM27] This allows for a wealth of varied information that can be used in a number of ways to complement the understanding of our own biology and answer questions about how growth factor signaling has evolved and what mechanisms of signaling and regulation are possible.

Some invertebrate FGF studies have provided very specific functional information. But many studies in recently sequenced models are still based on inferences from expression patterns or simply the presence of homologous domains in the genome. Much work remains to be done to complete the details of the complex signaling and regulatory networks that are present in FGF signaling to orchestrate the grand events of embryogenesis.

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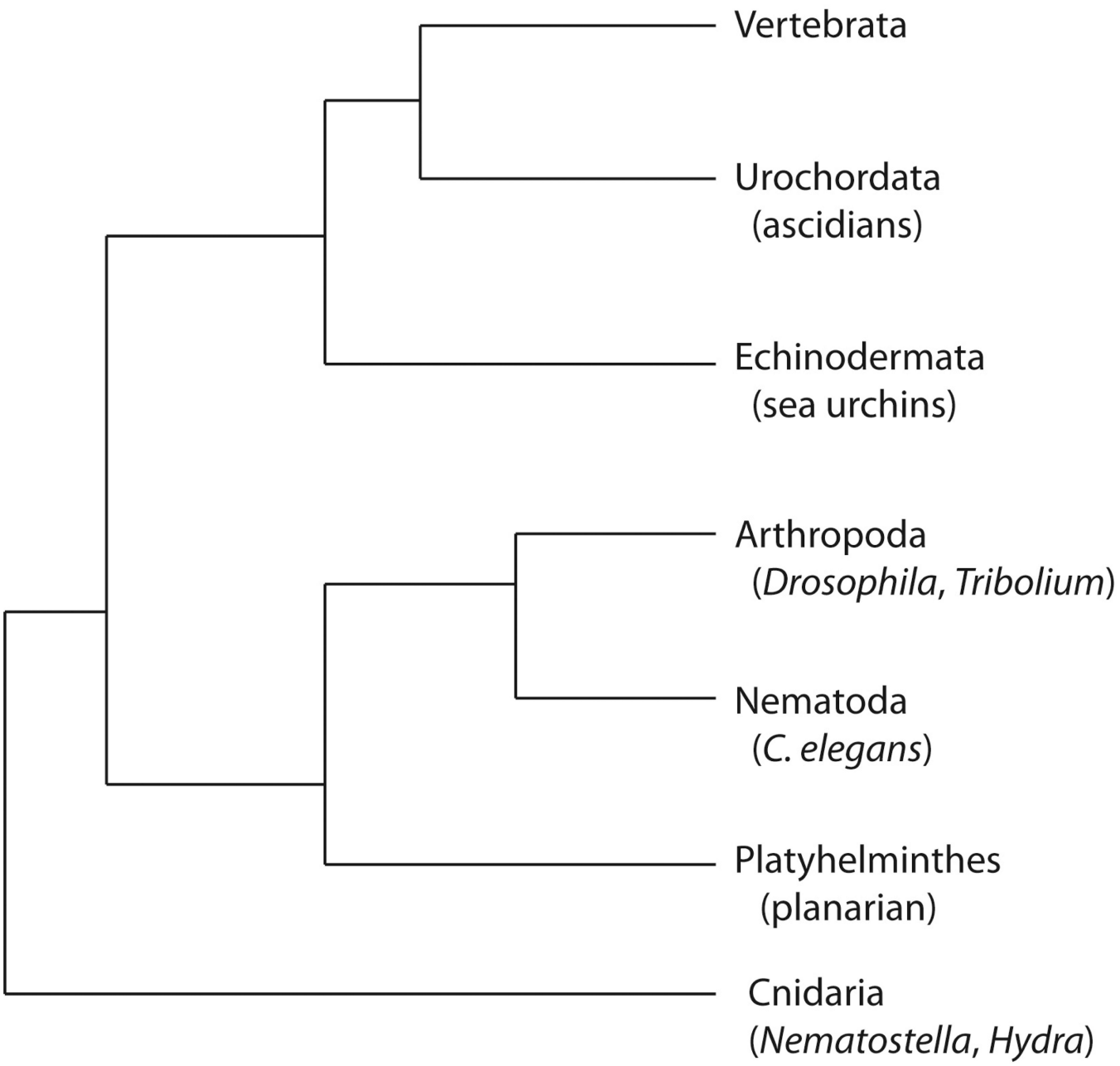

Figure 1.
Simplified tree of Metazoa phyla containing species discussed in this review.

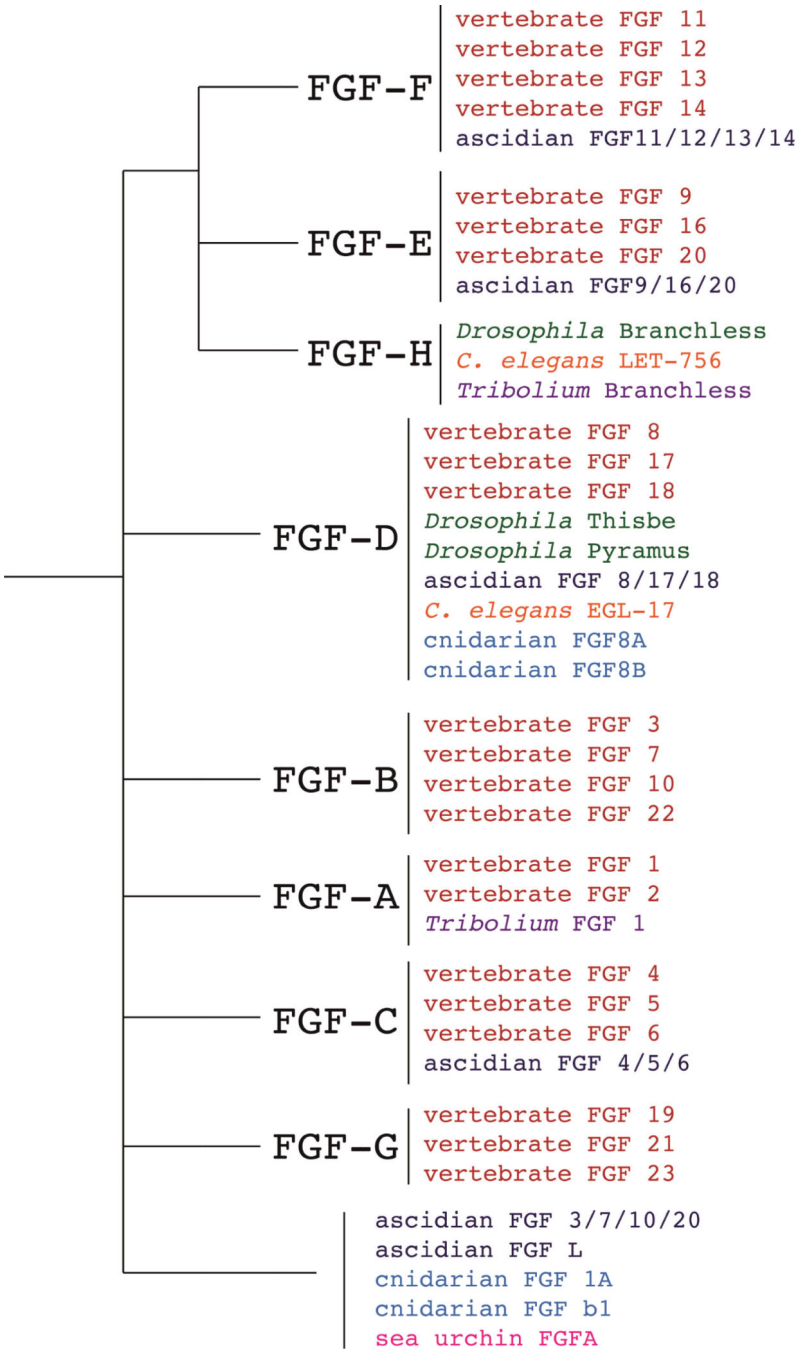

Figure 2.

Table

	aa	kD	known functions	expressed in	references
Ciona					
Ci-Fgf9/16/20	297	33	mesenchyme specification, dorsal midline formation, neural induction, heart specification, trunk ventral cell migration	vegetal hemisphere, endoderm, next to B7.5 cells	Miyazaki et. al, 2007; Tokuoka et. al, 2004; Pasini 2006; Betrand et. al, 2003; Beh et. al, 2007; Davidson et. al, 2006; Imai et. al, 2002; Kim et. al, 2000
Ci-Fgf8/17/18	414	48	brain patterning, notochord formation, atrial placode formation	CNS, midbrain-hindbrain boundary, trunk lateral mesenchyme	Ikuta et. al, 2007; Imai et. al, 2002; Yasuo et. al, 2007; Imai et. al, 2009; Kourakis and Smith, 2007
Ci-Fgf3/7/10/22	268	31	notochord convergent extension	floor plate	Shi et. al, 2009
Ci-Fgf4/5/6	229	27	?	?	?
Ci-11/12/13/14	182	21	?	?	?
Ci-FgfL	658	75	?	?	?
S. purpuratus					
FGFA	348	39	migration and differentiation of PMCs	lateral ectoderm	Rottinger et. al, 2008
Drosophila					
Branchless	770	84	tracheal branching, male genital disc development	cells surrounding developing tracheal branches, male genital disc	Sutherland et. al, 1996; Wolf et. al, 2002; Ribeiro et. al, 2004; Ahmad et. al, 2002
Pyramus	766	87	mesoderm collapse & intercalation; pericardial cell specification, axonal migration	lateral ectoderm, glial cells	Stathopoulos et. al, 2004; Klingseisen et. al, 2009; Franzdóttir et al., 2009; Kadam et. al, 2009; Gryzik et. al, 2004; McMahon et. al, 2010
Thisbe	748	82	mesoderm collapse & intercalation, mesoderm differentiation, glial differentiation	ventro-lateral ectoderm, neurons	Stathopoulos et. al, 2004; Klingseisen et. al, 2009; Franzdóttir et al., 2009; Kadam et al, 2009; Gryzik et. al, 2004; McMahon et. al, 2010
Tribolium					
Tc-FGF8	229	27	presumably brain patterning, mesoderm migration and differentiation	early posterior pole, ectodermal stripes, foregut anlagen, CNS midbrain-hindbrain boundary	Beerman and Schroder, 2008
Tc-Branchless	232	25	likely trachael, air sac and gland formation	between abdominal segment 10 and growth zone, growth zone, leg forming region, tracheal placodes, maxillary domain, anal ring	Beerman and Schroder, 2008
Tc-FGF1a	?	?	?	ubiquitous	Beerman and Schroder, 2008
Tc-FGF1b	?	?	?	ubiquitous	Beerman and Schroder, 2008
C. elegans					
Egl-17	216	25	migration of sex myoblasts, essential, regulation of fluid balance, axonal migration, negative regulation of muscle membrane extension	primary vuval cell secreted and nuclear, muscles adjacent to hypodermis	Burdine et. al, 1997; Burdine et. al, 1998 Roubin et. al, 1999; Popovici et. al, 2006; Dixon et. al, 2006

	aa	kD	known functions	expressed in	references
LET-756	425	50			
Nematostella					
Nv-FGF8A	?	?	?	invaginating blastopore, developing pharnyx, ectodermal cells of apical tuft	Matus et. al., 2007
Nv-FGF8B	?	?	?	endodermal cell at aboral end below apical tuft	Matus et. al., 2007
Nv-FGF1A	?	?	apical ciliary organ development	aboral pole, aboral ectoderm at base of apical tuft	Matus et. al., 2007; Rentzsch et. al, 2008
Nv-FGFa2	?	?	apical ciliary organ development	aboral pole	Rentzsch et. al, 2008