ANALYSIS OF DROSOPHILA FIBROBLAST GROWTH FACTOR FUNCTIONAL DOMAINS

Thesis by

Sarah L. Tulin

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"Forming an embryo is the hardest thing you will ever do. To become an embryo you had to build yourself from a single cell. You had to respire before you had lungs, digest before you had a gut, build bones when you were pulpy, and form orderly arrays of neurons before you knew how to think" ~Scott Gilbert, Developmental Biology 8th ed.

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ABSTRACT

The exciting Fibroblast Growth Factor (FGF) field lies at the crossroads of cell signaling, development, evolution, trafficking, physiology and human disease. A current challenge is to understand the mechanisms used by this signaling pathway to accomplish its myriad tasks in patterning the embryo, forming organs, and maintaining systems in the adult animal. My thesis work has focused on tackling this challenge in the model system of *Drosophila melanogastor*, the vinegar fly. By examining functional domains of Thisbe and Pyramus, FGF ligands in the fly, we have begun to understand the properties of *Drosophila* FGFs and the way in which they may contribute to regulation of FGF signaling.

FGF ligands in vertebrates are small molecules that bind to a corresponding receptor through two immunoglobulin domains. The FGF ligands in Drosophila are predicted to be much larger molecules than their vertebrate homologs. Whether Drosophila FGFs bind to the receptor as full-length proteins or are first cleaved to smaller molecules was previously unknown. My thesis work addressed this question through experiments in *Drosophila* embryos and *Drosophila* cell culture. I found evidence that the N-terminal FGF-domain alone is capable of signaling by itself in the embryo. In addition, experiments in cell culture showed that Thisbe and Pyramus are secreted as small forms, presumably as a result of intracellular proteolytic cleavage. Cleaved forms for Thisbe and Pyramus were detected in embryonic extracts as well. The Ths ligand is also present outside the cell as a full-length form and this form may act to regulate the diffusion or activity of the ligand. Addition of the Thisbe C-terminus to the Pyramus N-terminus to make a Pyramus-Thisbe chimeric protein creates a protein that has reduced activity compared to Thisbe alone. The

opposite Thisbe-Pyramus chimera creates a protein that has increased activity compared to Ths alone.

Over the course of animal evolution the FGF superfamily has diversified in many ways. Understanding the mechanism of FGF signaling in *Drosophila* and comparing this to other Drosophilids, insects, and more distantly related animals will reveal the likely makeup of the ancestral FGF signaling system.

TABLE OF CONTENTS

Chapter 1

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INTRODUCTION TO FIBROBLAST GROWTH FACTOR SIGNALING

One of the most basic needs for cells in a developing embryo is to be able to communicate with each other to coordinate growth and morphogenesis. Intercellular communication is achieved through cell-to-cell signaling. One type of cell signaling involves ligand molecules being sent from one cell and received by receptor proteins on another cell.

Cell signaling by Fibroblast Growth Factors (FGF) is essential to the development and maintenance of animals. From their discovery to today, researchers have continued to uncover the details of how FGF signaling contributes 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 the developing embryo and the adult. One way FGF signaling has adapted to these many varied roles is through the subfunctionalization of many homologous ligands created and lost in multiple rounds of gene and genome duplication and by expanding the possible ligand-receptor combinations through splice variants of both receptors and ligands. Complex regulatory steps have also evolved to mediate the effects of FGF signaling, which is highly potent. As more genomes are sequenced and more FGF superfamily members are found, 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 (detailed further in Chapter 4).

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 come for another 34 years. In 1973, Hugo Armelin showed a factor in pituitary extracts could stimulate growth of 3T3 mouse fibroblast cells and he characterized the molecule as being thermolabile, sensitive to proteases and enhanced by hydrocortisone (ARMELIN 1973). In 1974, Denis Gospodarowicz purified the mitogenic factor from pituitary extracts and found it was also likely 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}. A year later Gospodarowicz and Moran found 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 kDa molecule and was called basic FGF (bFGF) because of its high isoelectric point (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 as compared to bFGF (MACIAG *et al.* 1979).

A number of other mitogenic proteins named for the cell types they had activity on 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). More members of the FGF family were found using a variety of approaches and eventually 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 sequence homology to FGF4 (MARICS *et al.* 1989). FGF7 was found as a keratinocyte-specific growth factor (KGF) by classical protein purification from human embryonic lung fibroblasts and importantly, this study showed for the first time that FGFs are necessary for tissue homeostasis by enabling communication between mesenchymal and epithelial compartments (RUBIN *et al.* 1989). FGF8 was discovered as an androgen-induced growth factor (TANAKA *et al.* 1992). FGF9 was found to be able to stimulate 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 intracellular FGFs that were found by searching cDNA databases for sequences with homology to the conserved core region of FGF (ITOH and ORNITZ 2008; LAEZZA *et al.* 2009; ORNITZ and ITOH 2001a). It has since been found that these iFGFs are not secreted and do not bind to FGFR1-4, but instead to an intracellular kinase scaffold protein, islet brain-2 (IB2)

(COULIER *et al.* 1997; SMALLWOOD *et al.* 1996). iFGFs can also bind to heparin with high affinity like the canonical FGFs, yet despite striking structural similarity, iFGFs 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 as a member of the Tyrosine Kinase family of receptors (LEE *et al.* 1989; OLWIN and HAUSCHKA 1986), and since then 4 FGFRs have been found in vertebrates (COUMOUL and DENG 2003a). FGFR1 is expressed almost exclusively in the mesoderm and is essential to mediating early developmental functions and organogenesis (DENG *et al.* 1994). FGFR2 is detected mainly in epithelial lineages during gastrulation and later during organogenesis mediates reciprocal signaling between FGFs in the epithelium and mesenchyme. FGFR2 mutants uncouple the reciprocal regulatory loop between FGF8 and FGF10 in limb development (XU *et al.* 1998). FGFR3 is expressed mainly in the central nervous system and bone rudiments where it is a negative regulator of bone development (COLVIN *et al.* 1996; DENG *et al.* 1996). FGFR4 is expressed in the definitive endoderm and somatic myotome and cooperates with FGFR3 to control lung development and liver functions (WEINSTEIN *et al.* 1998).

Structure of FGF ligands and receptors

Most of the work on the structural characteristics of FGF ligands, receptors, binding to heparin compounds, mechanisms of dimerization and signal transduction has been carried

out on vertebrate FGFs 1-23 (MOHAMMADI *et al.* 2005). 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. Additionally, they have variable length N- and C-terminal tails, which largely account for the specific biology of different FGF family members. Most FGFs (except for FGF1, 2,9,16,20) have traditional signal peptides and are secreted as soluble signaling molecules. Vertebrate FGFs are also known to bind to polysaccharide-protein complexes called 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. Invertebrate FGFs are also thought to require heparin sulfate proteoglycans (HSPGs) for activity (LIN and PERRIMON 2000).

FGF ligands bind to the FGFR family of tyrosine kinase receptors in an 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 (Figure 1). The D1 and acid box are thought to play a role in receptor autoinhibition.

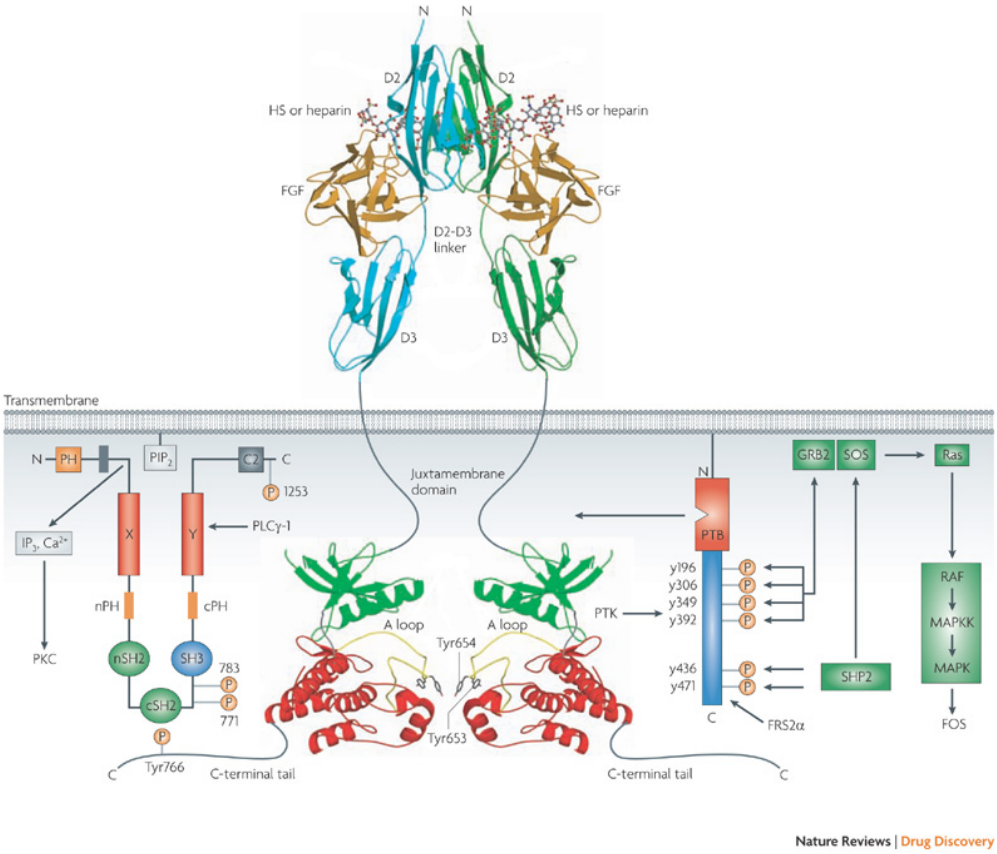

Figure 1 | Structure of Fibroblast Growth Factor receptor (FGFR) signaling. Figure from (BEENKEN and MOHAMMADI 2009). The structure of the receptor-ligand-HSPG complex is shown, with the HSPG fitting in the "basic canyon" and the FGF ligands binding to both the HSPG and the FGFR in between the D2 and D3 domains. The intracellular domains of FGFR are shown as well along with the major downstream signaling pathways utilized, which will be covered in the next section. The FGF ligands are shown by gold ribbons, the FGFR is shown in blue, green and red ribbons, and the HSPG is represented with a ball-and-stick model.

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 positively-charged "basic canyon" and contributes to dimerization by binding both the ligands and the receptors (Figure 1) (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 on A loop tyrosines to become activated. A loop phosphorylation results in phosphorylation of tyrosines in the C tail, the kinase insert and juxtamembrane regions (Figure 1) (MOHAMMADI *et al.* 1996).

Signal Transduction Pathways Downstream of FGFR

The phosphorylation of FGFRs triggers the activation of cytoplasmic signal transduction pathways. In addition to the catalytic core domain, the cytoplasmic domain of FGFR contains several regulatory sequences. The juxtamembrane domain of FGFRs is considerably longer than that of other receptor tyrosine kinases, and this region contains a highly conserved sequence that serves as a binding site for phosphotyrosine binding domains of the two members of the fibroblast growth factor receptor substrate 2 (FRS2) family of docking proteins: FRS2α and FRS2β (ESWARAKUMAR *et al.* 2005; THISSE and THISSE 2005). FRS2α contains four binding sites for the growth factor receptor-bound

protein 2 (Grb2), an adapter protein, and two binding sites for src homology protein 2 (Shp2), a protein tyrosine phosphatase. FGFR-stimulation leads to tyrosine phosphorylation of Shp2 resulting in complex formation of additional Grb2 molecules. Grb2 and its associated nucleotide exchange factor son-of-sevenless (Sos) are thus recruited directly and indirectly via Shp2 upon phosphorylation of FRS2α (BÖTTCHER and NIEHRS 2005). Grb2/Sos recruit and 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 that will affect FGF target genes. One of the transcription factors used by FGF signaling in *Drosophila* is the Ets transcription factor. FRS2α can also be used to degrade FGF receptor molecules to result in signal attenuation and fine-tuning of activity by recruiting negative regulators (ESWARAKUMAR *et al.* 2005). FRS2α/Grb2 can form a ternary complex with Cbl, which results in ubiquitination of FGFR and FRS2α. Cbl is a multidomain protein that posseses an intrinsic ubiquitin ligase activity.

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 three ways after activation of FGFR (BÖTTCHER and NIEHRS 2005). First, Gab1 can bind to FRS2 indirectly via Grb2, resulting in tyrosine phosphorylation and activation of the PI3-kinase/Akt pathway via p85. Second, the PI3 kinase-regulatory subunit p85 can bind to a phosphorylated tyrosine residue of the FGFR, which was shown in *Xenopus* cell extracts and the in the *Xenopus* embryo where a dominant negative form of the p85 subunit interfered with mesoderm formation. Finally, activated Ras can induce membrane localization and activation of the p110 catalytic subunit of PI3 kinase.

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. Further studies will be necessary to pinpoint which downstream pathways are used in specific contexts and to understand how specific ligand-receptor combinations trigger specific downstream pathways.

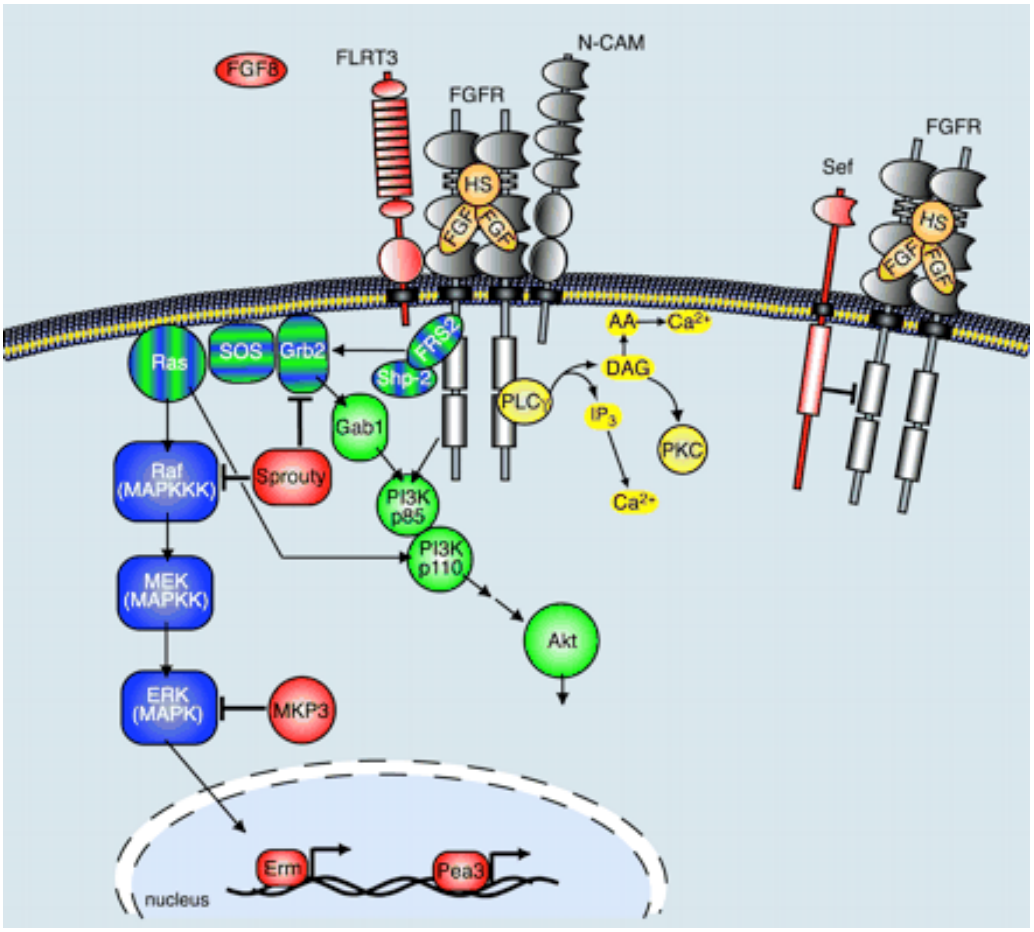

Figure 2 | Intracellular signaling pathways activated through FGFRs. Figure from (BÖTTCHER and NIEHRS 2005). Receptor autophosphorylation and activation of intracellular signaling cascades, including the Ras/MAPK pathway, PI3 kinase/Akt pathway, and the PLCγ/Ca^{2+} pathway. Proteins in two pathways are *striped*.

FGF Developmental Functions in Vertebrates

Research on the functions of the FGF signaling superfamily in the last 36 years has unveiled crucial functions of FGF signaling in developing embryos from many different animals. FGFs are key regulators of developmental processes including mesoderm induction, gastrulation, cell migration, midbrain-hindbrain patterning, limb development,

heart development and bone formation. Specific FGF ligands are produced in either epithelial or mesenchymal tissue and usually activate receptors expressed in the opposite tissue type; i.e. normally, a ligand produced in the epithelium will activate a mesenchymal receptor and vice versa. Additionally, alternatively spliced receptor variants will be expressed in either the ectoderm or mesoderm and only respond to ligands expressed in the opposite germ layer.

Gastrulation

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. This disruption of migration leads to the loss of embryonic mesoderm- or endoderm-derived tissues, but extraembryonic tissues still form (SUN *et al.* 1999; THISSE and THISSE 2005). FGFR1-/- mouse mutants are also gastrulation defective and embryonic lethal with severe reductions in paraxial mesoderm formation and an expansion of axial mesoderm (CIRUNA *et al.* 1997; DENG *et al.* 1994; SUN *et al.* 1999; YAMAGUCHI *et al.* 1994). FGFR1 orchestrates the epithelial-to-mesenchymal transition and morphogenesis of the mesoderm at the primitive streak by controlling expression of *snail* and E-cadherin (CIRUNA and ROSSANT 2001).

FGF induction of mesoderm has also been studied in *Xenopus laevis,* where basic FGF (FGF2) was first shown to have mesoderm inducing activity equivalent to the ventrovegetal signal (SLACK *et al.* 1987). Interestingly, in this case, heparin inhibits the binding of bFGF to its receptor and reduces the effectiveness of bFGF as an inducer. More recently, the

specific roles of different splice forms of FGF8 on mesoderm induction were examined. 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. Human FGF8b and mouse Fgf8f also had similar activities to *X. laevis* FGF8b (FLETCHER *et al.* 2006).

Limb Patterning

The development of the vertebrate limb has been very well studied and FGFs have been found to play key roles in the process. Vertebrate limbs grow out from a bud of thickened lateral plate mesenchyme. Three key areas are then formed with specialized growth and patterning functions: the AER, the progress zone and the ZPA. The ectoderm surrounding the distal tip of the bud is induced by the mesenchyme to become a specialized structure, the apical ectodermal ridge (AER). The mesenchyme underlying the AER is termed the progress zone, which contains undifferentiated precursor cells. Additionally, proximal and posterior to the progress zone lies the zone of polarizing activity (ZPA), responsible for setting up the anteroposterior limb axis (the thumb to little finger in humans).

Formation of limb buds and their successful outgrowth is dependent upon FGF signaling and a FGF positive-feedback signaling loop between the limb mesenchyme and the overlying ectoderm. Removal of the AER causes a cessation of growth and truncation of limb, resulting in a varying amount of limb structures depending on when the AER was removed (SAUNDERS 1948; SUMMERBELL 1974). This result led to an initial hypothesis

termed the progress zone model that described the progressive development of more distal limb structures.

FGFs are the key molecules mediating the activity of the AER. In 1993, Niswander and colleagues found that placing two heparin-linked FGF4 beads at the ZPA and apical mesenchyme, following removal of the AER, restored development of all limb structures, although the digits were still abnormal. These results lead to the hypothesis that FGFs could induce both growth and the polarizing activity.

FGF4, FGF8, FGF9 and FGF17 are all expressed in the AER. Recent combinatorial mutant studies resulted in the loss of intermediate skeletal structures while the most distal and the most proximal structures remained intact. This has led to a new hypothesis termed the 'two-signal model,' which describes limb mesenchyme initially being influenced by one signal (likely Retinoic Acid) that influences proximal cell fates and while the distal domain is established by FGF signals from the AER. The intermediate domain would then form as a result of interactions at the boundary between the distal and proximal domains.

Sonic hedgehog (Shh) is expressed in the ZPA and is thought to be responsible for anterior/posterior patterning. 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).

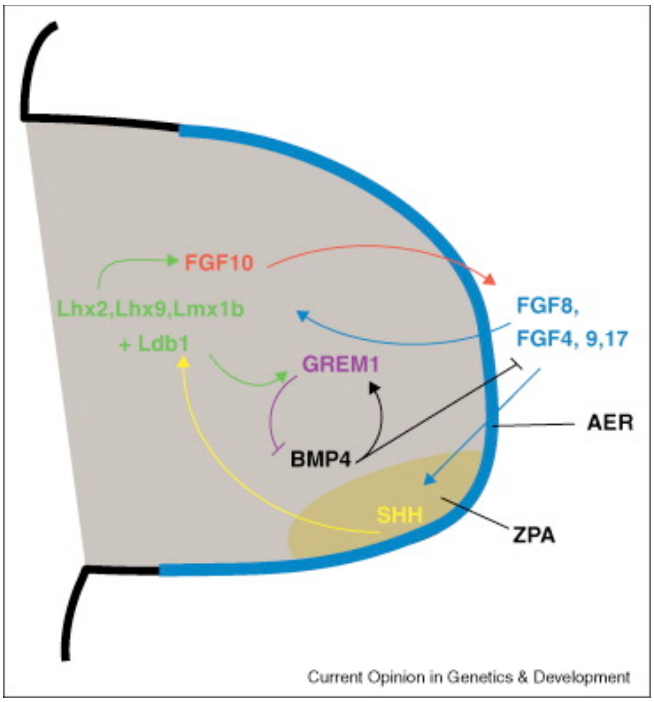

Figure 3 | Limb Patterning. Figure from (DUBOC and LOGAN 2009). The zone of polarizing activity (ZPA) is in the most posterior position in the limb bud and expresses sonic hedgehog (SHH). FGFs from the AER signal to the proximally located progress zone and establish a feedback loop and also signal to the ZPA. Lhx2, Lhx9, Lmx1b are Lim-domain homeobox transcription factors that, along with their cofactor Ldb1, mediate signal integration and feedback loops in the developing limb.

Mid-hindbrain Patterning

Patterning of the midbrain-hindbrain (MHB) analage depends on the activity of an organizer 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 from a mouse with a severe hypomorphic allele of *Fgf8* (MEYERS *et al.* 1998). Conditional Cre-mediated mutants were subsequently used to rule out the role of FGF8 in gastrulation and it was determined that FGF8 in the Isthmus is part of a gene regulatory network essential for cell survival and cerebellum proliferation (CHI *et al.* 2003). FGF17 and FGF18 are also expressed in the mid/hindbrain in a broader domain than that of FGF8 that includes posterior midbrain (MARUOKA *et al.* 1998). The loss-of-function of FGF17 in the mouse results in the truncation of posterior midbrain and reduced proliferation of the anterior cerebellum. 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 the expression of other marker genes: *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).

FGF8 is differentially spliced to generate FGF8a and FGF8b isoforms, which are both expressed at the Isthmus/MHB and they differ in only 11 amino acids that are included in FGFb (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 fate (LIU *et*

al. 1999). After FGF8b is induced in the presumptive rhombomere1 territory it induces FGF18 in the surrounding tissue. 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). Interestingly, it was also determined that the *Fgf8b* signal is 100 times stronger than the *Fgf8a* signal (SATO *et al.* 2001). Loss-of-function studies confirmed the relative importance of the FGF8 spliceforms and FGF8a mutants had no discernable defect in the midbrain and cerebellum (GUO *et al.* 2010).

In Zebrafish, FGF8 is also present at the MHB and acts as a morphogen to pattern the midbrain. A mutant called *acerebellar* in which FGF8 is missing its second exon and prematurely stopped, lacks a functional MHB and also lacks a cerebellum (REIFERS *et al.* 1998). Studies in zebrafish embryos showed that endocytosis is a likely mechanism controlling the rate of FGF8 diffusion (SCHOLPP and BRAND 2004). Inhibition of internalization causes FGF8 to accumulate extracellularly, spread further, and activate target genes over a greater distance. Enhanced internalization increases FGF8 uptake and shortens its effective signaling range. The mechanism of FGF8 diffusion was further explored by tracking GFP-tagged FGF8 molecules with fluorescent correlation spectroscopy (FCS) as they diffuse into their target tissue (YU *et al.* 2009a). The results support a simple source-sink mechanism as the driving force behind setting up the FGF8 gradient where uptake by the target cells is regulated by receptor-mediated endocytosis. Additionally, HSPG-linked FGF8 molecules were found to diffuse slower, indicating a role for HSPGs in restricting the signaling range of FGF proteins.

Bone Formation

Bones form through two major processes: intramembranous ossification and endochondral ossification. Intramembranous ossification occurs when mesenchymal cells directly differentiate into osteoblasts. All flat bones, including the calvarias bones of the skull, use intramembranous ossification. In endocondral ossification, mesenchymal cells first differentiate into cartilaginous tissue and later the cartilage is replaced by bone. Bones of the vertebral column, face, medial clavicles and the long bones of the limb are all formed through endocondral ossification.

FGF signaling is capable of regulating genes at all steps of osteogenesis. The involvement of FGFs in bone formation was first realized when a point mutation in the transmembrane domain of FGFR3, causing a dominant mutation, 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). The activity of FGF signaling is dependent on the spatiotemporal pattern of expression of FGFRs including FGFR1 and FGFR2 which are in mesenchyme during condensation prior to deposition of bone matrix at early stages of long bone development and in cranial structures. 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.

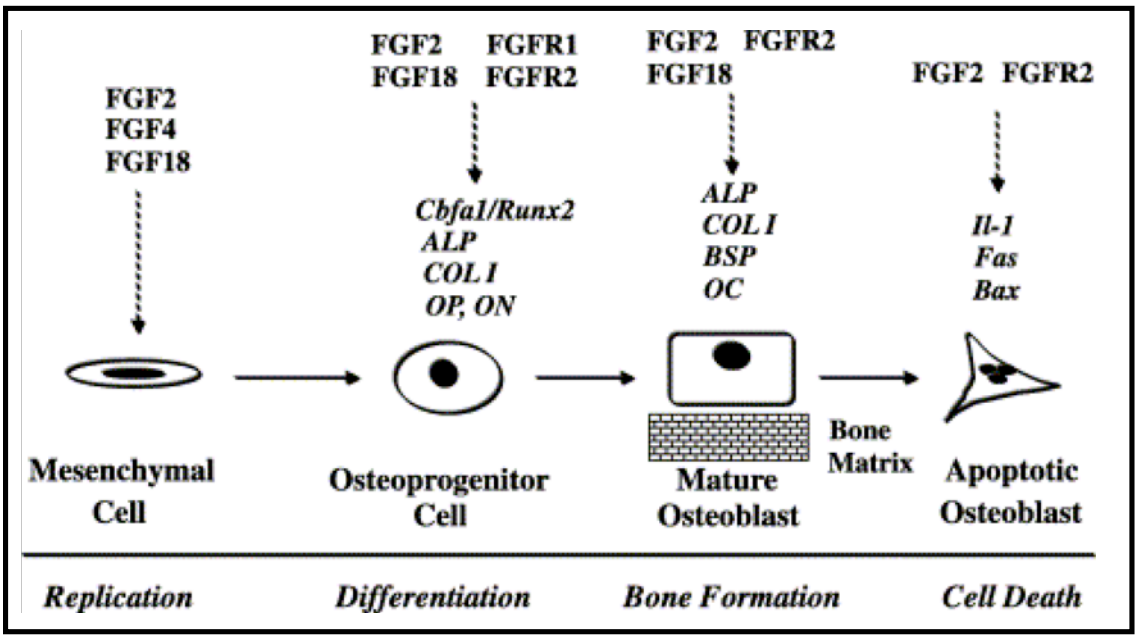

Figure 4 | FGFs in Bone Development. Figure from (MARIE 2003). Depiction of the role of FGFs at all stages of bone formation. Osteoblasts develop from mesenchymal cells that are induced to turn on the Runx2 transcription factor and begin the transition to mature osteoblasts.

Other Developmental Functions in Vertebrates

Other developmental functions for FGFs in vertebrates have been described as well, including: neural induction, epidermis development, lung development, mammary glad development, central nervous system development, heart development, ear development,

kidney development, liver development, pancreas development (COLEMAN-KRNACIK and ROSEN 1994; DELAUNE *et al.* 2005; KOBBERUP *et al.* 2010; LAVINE and ORNITZ 2008; LAVINE *et al.* 2005; SAWADA *et al.* 2001; THISSE and THISSE 2005; WILKIE 2005).

FGF Signaling in Drosophila

In vertebrates, the large number of FGF ligands, the varied range of their activities and complex signaling networks have made the precise understanding of FGF signaling difficult. *Drosophila* is a model system with reduced cellular and anatomical complexity compared to mammals and its genetics are very well understood and easily manipulated. The FGF/FGFR network is also much less complex in *Drosophila*, which allows for different insights.

Discovery of Breathless and Heartless receptors

The study of FGF signaling in *Drosophila* began with the discovery of the *breathless* (*btl*) FGFR gene in 1992 (KLÄMBT *et al.* 1992). A second FGFR was found 4 years later in 1996 and called *heartless* (*htl*) (BEIMAN *et al.* 1996; GISSELBRECHT *et al.* 1996). To date, one ligand has been found for the *btl* receptor and two ligands have been found for the *htl* receptor. This brings the current totals for the FGF family in *Drosophila melanogastor* to two receptors and three ligands. No other ligands or receptors are predicted in the current genome annotation.

Branchless ligand

Branchless (*bnl*), the ligand for *btl*, was discovered in 1996 (SUTHERLAND *et al.* 1996). Together, *bnl* and *btl* control the branching of the developing trachea and glial migrations

in the developing CNS. *Bnl* is expressed in clusters of epidermal cells that surround the branching tracheal tube. These clusters prefigure where branches will form. The expression of *bnl* is highly dynamic, turning off in some cells and on in others rapidly to accommodate the generation of secondary and tertiary branches. It appears that *bnl* is a chemoattractive molecule that guides the formation of trachea in the correct position as localized misexpression of *bnl* can cause the formation of branches in ectopic positions. Studies of *btl* mutants also revealed a phenotype in the CNS. In *btl* mutants the posterior pair of midline glial cells do not complete their migration toward the posterior commissure of the adjoining segment (KLÄMBT *et al.* 1992). In both the developing trachea and the developing CNS signaling through *bnl/btl* is important for correct cell migration.

Pyramus and Thisbe ligands

Signaling through the *htl* FGF receptor was studied for eight years before the ligands for the receptor were found. Traditional searches for the ligands failed due to the partially overlapping functions of two FGF ligands and because there was a mistake in the *Drosophila* genome annotation that omitted the first exon, which contains the FGF-homologous domain. One ligand for the Htl receptor was identified in a microarray screen for targets of the Dorsal (Dl) transcription factor present in the neurogenic ectoderm and was originally known as Neu4 (STATHOPOULOS *et al.* 2002). Subsequent analysis of Neu4 revealed it was most likely a ligand for Htl and an additional, related ligand was then found as well. These ligands were named Thisbe (originally Neu4) and Pyramus after the heartbroken lovers described in Ovid's Metamorphoses (STATHOPOULOS *et al.* 2004). The same genes were also identified in a screen of large deficiency mutants that affect the

migration of the mesoderm during migration and called FGF8-like1 and FGF8-like2 due to their similarity to the vertebrate FGF ligand FGF8 (GRYZIK and MÜLLER 2004b).

The *htl* mutant phenotype is pleiotropic but the most striking defect is the massive loss of heart cells as revealed by the markers Eve (antibody against the Even-skipped protein), DMef2 (expressed in all myogenic lineages) and Ab.3 (a monoclonal antibody recognizing all pericardial cells) (BEIMAN *et al.* 1996). The earliest defect is a failure of the mesoderm to undergo its usual dorsolateral migration along the ectoderm to create a monolayer. It is thought this migration is crucial to later differentiation of the mesoderm because only cells reaching the dorsolateral margin are able to receive the differentiating Decaplentaplegic (Dpp) signal from the dorsal ectoderm. Ectopic activation of Dpp in *htl* mutants partially rescues the loss of visceral and cardiac mesoderm, as determined by expression of *bagpipe* (*bap*). The *bap* gene is normally restricted to segmentally repeated patches of dorsal mesoderm, but when *dpp* is expressed throughout the mesoderm, *bap* is seen throughout the ventral mesoderm (GISSELBRECHT *et al.* 1996).

Double mutants for *thisbe* (*ths*) and *pyramus* (*pyr*) [Df(2R)BSC25] phenocopy the *htl* mutant phenotype (STATHOPOULOS *et al.* 2004). The early mesoderm, visualized with an antibody to the Twist protein, which marks all the mesoderm, does not migrate to form a monolayer, but remains in a clump after invaginating through the ventral furrow and forming an epithelial tube. Df(2R)BSC25 and *htl*- embryos also have lost all Eve-positive pericardial cells at stage 11.

Subsequently, individual mutants for *ths* and *pyr* were generated to identify the individual and overlapping functions of these ligands thought to have originated through an ancient dipteran-specific gene duplication (KADAM *et al.* 2009; KLINGSEISEN *et al.* 2009a). Both ligands were found to influence mesoderm spreading, whereas *pyr* is the dominant player controlling Eve-positive cell specification in the dorsal mesoderm. The precise spatial positions of the ligands were also found to be important for their function. In contrast to vertebrate FGF biology where many ligands use the same receptor, it was found that *bnl* could not act in *htl*-dependent functions (KADAM *et al.* 2009).

Specification of pericardial cells

One of the key functions of signaling through Htl is the specification of the mesodermally derived pericardial cells, which are non-contractile cells that support the myocardial cells of the heart tube. These cells derive from dorsal mesodermal cells that express Eve. Each embryonic hemisegment contains a pair of Eve-expressing pericardial cells (EPCs) and a single Eve-positive dorsal somatic muscle, DA1, (FRASCH and LEVINE 1987) for a total of three Eve-positive cells per hemisegment. The two EPCs come from a common progenitor, P2, whereas a second progenitor, P15, gives rise to muscle DA1 (BUFF *et al.* 1998; CARMENA *et al.* 1998b). P2 develops from cluster 2 (C2) and comprises the dorsalmost cells of the preC2 precluster. The formation of EPCs and DA1 is dependent on multiple signaling pathways, including Wg, Dpp, EGF and FGF (FRASCH 1995; LAWRENCE *et al.* 1995; MICHELSON *et al.* 1998b; PARK *et al.* 1996; WU *et al.* 1995) and the expression *lethal scute* (*l'sc*), a neurogenic gene (CARMENA *et al.* 1995).

Previously, it was unclear if Htl was necessary for mesodermal cell fate specification or if it was only important for mesoderm migration. Htl was shown to play a direct role in mesodermal cell fate specification when a dominant negative form of Htl was expressed in the embryonic mesoderm and although migration was normal, there was loss of the C2 cluster, leading to loss of Eve-positive cells (CARMENA *et al.* 1998a).

The ligand thought to be primarily responsible for activation of Htl during Eve-positive cell specification is Pyr. *Pyr* transcripts are present in the ectoderm overlying the differentiating mesoderm during this stage, whereas *ths* transcripts are not (STATHOPOULOS *et al.* 2004). Analysis of single mutants of *ths* and *pyr* revealed that loss of *pyr* resulted in abnormal numbers of Eve-positive clusters (KADAM *et al.* 2009; KLINGSEISEN *et al.* 2009b). However, overexpression of both *pyr* and *ths* ectopically in the ectoderm led to supernumerary Eve-positive cells, indicating that although *ths* is not normally expressed in this location, it can still bind Htl in this context and affect Eve cell specification (KADAM *et al.* 2009).

Glial cell migration and axonal wrapping in eye disc

Within the *Drosophila* eye imaginal disc, FGF signaling through Htl coordinates glial proliferation, migration and then axonal wrapping (FRANZDÓTTIR *et al.* 2009). Glial cells originate from a pool of central-nervous-system-derived progenitors and migrate onto the eye imaginal disc where they switch from glia-glia interactions to glia-neuron interactions and then form a glial membrane around axonal trajectories. *Htl* expression is found in the eye disc glia and most strongly at the front of the migratory glial cell population. Htl protein is expressed widely in the glia and decorates glial projections following the

photoreceptor axons. A dominant-negative form of Htl or RNAi against *htl* caused a 40% reduction in glial cell number, impaired migration and a lack of differentiation. Also, an activated form of Htl resulted in an eightfold increase in glial cell number and also impaired glial migration.

The switch from glial cell proliferation and migration to differentiation and axonal wrapping seems to be dependent on a switch in signaling from the Pyramus FGF ligand to the Thisbe FGF ligand. Expression of *pyr* is seen in the eye disc anterior to the morphogenetic furrow and the glia, whereas *ths* is only expressed in photoreceptor neurons. A hypomorphic *pyr* p-element allele leads to a 50% reduction in glial cell number (FRANZDÓTTIR *et al.* 2009). Ectopic *pyr*-expressing cells are able to direct glial cells to migrate across the morphogenetic furrow, which normally they never do. These data suggest Pyr initially acts as an auto- or paracrine signal to regulate glia cell number and then facilitates glial migration

Ths, on the other hand, seems to be important for stopping glial migration and induces differentiation and axonal wrapping. Loss of *ths* results in an overmigration phenotype for glial cells. A reduction of neuronal *ths* leads to a severe differentiation phenotype where photoreceptor axons form abnormal fascicles and are less associated with wrapping glial processes. Neuronal overexpression of Ths caused increased glial cell membrane formation (FRANZDÓTTIR *et al.* 2009).

The key to the different signals is complex because both Pyr and Ths can cause axonal hyperwrapping and neuronal expression of *pyr* in a *ths* mutant eye disc rescues the glial

wrapping phenotype. This is similar to the ability of Ths to cause additional Eve-positive cells during mesoderm differentiation even though Pyr is the ligand normally expressed in the ectoderm overlying developing heart cells and thought to be solely responsible for Htl-mediated FGF signaling at that time and location in development. Perhaps the different ligands do have different properties but when ectopically expressed they are able to compensate for the loss of the other ligand.

Specification of adult muscles

Signaling through Htl is also important for the specification of adult muscle fibers in *Drosophila* (DUTTA et al. 2005). Over-expression of dominant-negative Htl (UAS-*dnhtl*) in all adult myoblasts using the 1151-GAL4 driver resulted in a decrease in the number of muscle founder cells per hemi-segment. Complementarily, over-expression of an activated form of Htl (UAS-λ*htl*) with the same 1151-GAL4 driver led to an increased number of muscle founders and consequentially, an increased number of muscle fibers in the abdomen. The mechanism of localized *htl* activation seems to be dependent on a positive regulatory loop with the stumps activator and the action of inhibition by the Sprouty protein (see Sprouty under "Regulation of FGF signaling").

Recruitment of cells to developing male gonad

Another important function of FGF signaling in *Drosophila* that is conserved in vertebrates is the recruitment of cells to form the male gonad. In flies, *bnl* expression, likely controlled by the doublesex transcription factor, in the ectoderm-derived cells of the male gonad, recruits *btl*-expressing mesodermal cells into the disc where they transform into cells of

epithelial character that will eventually give rise to the paragonia and vas deferens (AHMAD and BAKER 2002). In the female gonad *bnl* expression is repressed by the female form of doublesex, Dsx^F. In vertebrates, FGF9 plays a similar role in the development of the male testis (COLVIN *et al.* 2001). In the absence of FGF9, XY mice undergo male-to-female sex reversal. FGF9 causes the migration of the mesonephric cells, which are also mesodermal in origin, into the male gonad and ectopic expression of *FGF9* can induce mesonephric cells into the female gonad. Ectopic expression of *bnl* is likewise sufficient to cause *btl*-expressing cells to migrate into the female primordium of a *dsx* disc.

Stumps / Downstream-of-FGF / Heartbroken

An important member of the FGF signaling cascade in *Drosophila* was found by three independent research groups, and called Stumps (IMAM *et al.* 1999) / Downstream-of-FGFR (Dof) (VINCENT *et al.* 1998)/ Heartbroken (Hbr) (MICHELSON *et al.* 1998a). Stumps mutant embryos have normal determination of tracheal cell fate and cell division but tracheal cells do not migrate and therefore the tracheal network fails to form. Additionally, in stumps mutants, the mesoderm does not spread normally on the underlying ectoderm and this leads to missing heart precursor cells, disrupted musculature, and an insufficient amount of visceral mesoderm. These phenotypes are the combination of *htl* and *btl* mutants, and therefore it is thought that Stumps is required for all FGF signaling in *Drosophila*.

Stumps is not expressed ubiquitously like other RTK signaling components including Ras and ERK. The expression pattern of *stumps* RNA and protein is identical to the combined expression patterns of the two FGFRs, revealing it is only present in the cells where FGFR-

mediated signaling will take place. *Htl* and *btl* expression is not affected in Stumps mutant embryos and *stumps* expression is not affected in Htl and Btl mutant embryos.

In order to position Stumps correctly in the FGF signaling pathway, activated forms of the receptors and activated Ras were used to see which could overcome the defects in Stumps mutants. Activated Ras is able to compensate for loss of Stumps, but activated Btl cannot, indicating that Stumps is downstream of the FGF receptor but upstream of Ras. A monoclonal antibody for the dual phosphorylated form for MAPK (dpMAPK) reveals high levels of activated MAPK at the leading edge of migrating mesoderm cells. This expression is dependent on *htl* and is completely gone in *htl* mutants. Activated Htl causes a low, uniform dpMAK signal throughout the mesoderm in *htl* mutants and is able to partially rescue mesoderm migration. This effect of activated Htl is dependent on Stumps, as dpMAPK signaling is gone when Stumps function is reduced and mesoderm migration is not rescued. These results are also consistent with Stumps function being upstream of MAPK.

Stumps is specifically required for FGFR signaling, as Stumps mutants do not have any defects in other RTK signaling pathways, like EGF signaling. This suggests that Stumps is a key molecule in mediating specificity of signaling through multiple RTKs during development. When the intracellular domains of Btl and Htl were replaced with the EGFR or Torso domains, signaling was no longer dependent on Stumps (DOSSENBACH *et al.* 2001).

Summary of Drosophila FGFs

Drosophila continues to be a model for functions and mechanisms of FGF signaling in invertebrates. There are other uses of FGF signaling in *Drosophila* that remain to be characterized in detail. For instance, it is known that Htl is involved in the migration of the caudal visceral mesoderm, but the details of this interaction are still being worked out (MANDAL *et al.* 2004). Work on FGFs is also being carried out in another invertebrate model, *C. elegans*.

FGF Signaling in *C. elegans*

FGF signaling has also been characterized in another important invertebrate model, the nematode, *Caenorhabditis elegans (C. elegans)*. *C. elegans* and a related species, *C. briggase,* have two ligands, LET-756 and EGL-17 and a single receptor, EGL-15 (BIRNBAUM *et al.* 2005). Despite the small number of FGF family members, several interesting properties of FGF signaling are present in this model, including alternative splicing of the receptor and nuclear localization of one of the ligands.

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. Goodman et al. (2003) have shown that the different isoforms mediate signaling through different modules, involving 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 (GOODMAN *et al.* 2003).

EGL-17 is a member of the FGF8/17/18 family. The *egl-17* gene is located on the X chromosome and encodes a protein of 216 amino acids and predicted molecular mass of ~25 kDa. EGL-17 is functionally important for the migration of the sex myoblasts (SMs) (BURDINE *et al.* 1997; LO *et al.* 2008; STERN and HORVITZ 1991). The SMs begin as a pair of muscle precursor cells born at the posterior of L1 larvae. In the hermaphrodite they migrate anteriorly to functional positions flanking the central gonad and developing vulva where they differentiate into uterine and vulval muscles required for egg laying. Mutations in EGL-17 (and EGL-15) cause the SMs to be severely posteriorly displaced. EGL-17 is expressed in developing gonad and vulva and is thought to serve as a chemoattractant to EGL-15-expressing SMs expressing the EGL-15(5A) while EGL-15(5B) has a repulsive function, blocking anterior migration in the absence of EGL-15(5A) (BURDINE *et al.* 1998; LO *et al.* 2008).

LET-756 is a member of the FGF9/16/20 family, which was determined not only by sequence similarity, but through 'functional phylogeny' where only the core of vertebrate FGF 9, FGF16, and FGF20 could replace the core of LET-756 and rescue lethality in a LET-756 mutant (POPOVICI *et al.* 2004). *Let-756* encodes protein of 425 amino acids and a predicted molecular mass of ~50kDa. Uniquely, LET-756 contains several nuclear localization signals (NLS) and an atypical secretion sequence dependent on a six-residue motif (EFISIA) and a Golgi-associated secretion mechanism also used by the FGF9 family in mammals (POPOVICI *et al.* 2004). Nuclear localization and secretion of LET-756 is balanced in wild type worms. This balance is disrupted in mutants lacking the various NLSs, a stretch of glutamines and histidines (POPOVICI *et al.* 2006). A severe loss-of-

function allele of *let-756* causes arrest in early larval stages, whereas a partial mutant that truncates the C-terminal quarter of the protein allows some worms to develop to adult stages (ROUBIN *et al.* 1999). LET-756 is involved in axon outgrowth at the ventral midline. In *C. elegans*, axons use a special substratum composed of hypodermis and muscle located at the midline of the ventral cord. LET-756 is produced by the muscle and signals to hypodermally expressed EGL-15(5B) to provide the necessary substratum for axon outgrowth. FGF signaling through LET-756/EGL-15 negatively regulates muscle membrane extension. Body wall muscles in the worm have plasma membrane extensions called muscle arms that are guided to the motor axons to form the postsynaptic element of the neuromuscular junction. Too little FGF signaling in this context results in ectopic membrane extensions and too much signaling prevents membrane extension (DIXON *et al.* 2006).

LET-756 is also involved in tissue homeostasis and fluid balance in the adult worm through paracrine signaling to EGL-15. Increased EGL-15 signaling leads to fluid accumulation and leads to a Clr phenotype, in which animals display accumulation of clear fluid within the pseudocoelomic space. Decreased EGL-15 signaling results in a Soc (suppressor of clr), Scr (scrawny, meager body size) or Let (lethal, animals die prematurely) phenotype (HUANG and STERN 2004).

Downstream RTK components are conserved in *C. elegans*, including SEM-5/GRB2, LET-341/SOS, LET-60/RAS, LIN-45/RAF, and MPK-1/MAPK. However, a homolog of FRS2 has not been found. Phospholipase C-γ or PLC-3 has a homolog in the worm but it may

not function in EGL-15 signaling. Specific to *C. elegans*, CLR-1 phosphatase negatively regulates FGF signaling through EGL-15, but has no equivalent in mammals.

The worm has managed to pare down its FGF repertoire to just two ligands and a single receptor, yet retains the seemingly vital functions of cell migration, axon growth and tissue homeostasis. Studying this relatively simple system in great detail has led to an interactome with many connections that may prove valuable to the larger FGF field (POLANSKA *et al.* 2009).

FGF Functions in the Adult

FGFs do not cease to be important when development is finished, but instead they continue to be vital to the adult animal. FGFs are involved in homeostatic regeneration, wound healing, hepatic function, angiogenesis and serum phosphate level regulation.

Vertebrate organs use homeostatic regeneration to regularly replace cells lost through apoptosis, daily wear and aging. Studies in zebrafish found that the same molecules that function during regeneration after injury are involved in homeostatic regeneration and daily cell turnover. Long-term inhibition of FGFRs in uninjured zebrafish led to the progressive loss of distal fin structures (WILLS *et al.* 2008). The specific ligand FGF20a was implicated in this process as it is expressed in the intact fin and mutants displayed the progressive loss of distal fin structures phenotype. FGF signaling has also been shown to play a major role in the maintenance of vascular integrity in the existing adult vasculature (MURAKAMI *et al.* 2008). Suppression of FGF signaling can be accomplished with a

dominant negative FGFR that inhibits signaling through all 4 FGFRs or with a soluble FGFR-IgGFc chimera that acts as a FGF trap. Suppression of FGF signaling results in the loss of endothelial cell-cell contact due to decoupling of p120-catenin from VE-cadherin and the subsequent disruption of adherens and tight junctions in both arteries and veins. Through these studies and others it is clear that adult animals depend on FGF signaling to maintain their adult structures through cell turnover and repair.

The role of FGFs in wound healing has focused on the ligands FGF2, FGF7 and FGF22. Kurita and colleagues examined the localization of FGF2 during wound healing in the skin and found that during mouse skin wound healing the basal layer keratinocytes and hair bulbs at the wound edge are strongly stained with anti-FGF2 antibodies (KURITA *et al.* 1992). Additionally, the role of FGF2 in wound healing was highlighted by the finding that FGF2 mutant mice have delayed wound healing as compared to their wild-type littermates (ORTEGA *et al.* 1998). FGF2 has been implicated in a wide range of activities, yet surprisingly FGF2 mutant mice are viable, fertile, and grossly indistinguishable from their wild type littermates. This finding points to a problem with trying to understand FGF function by ectopic expression alone because many times the ectopic FGF can interact with receptors it doesn't use *in vivo*. However, on the other hand, FGFs can function redundantly and therefore knocking out just one at a time will not reveal all the functions taken over by redundant FGFs. FGF2 was studied with both methods in the context of wound healing. Topical application of FGF2 accelerates healing of skin wounds, as well as of eye, retina and corneal wounds (BIKFALVI *et al.* 1997). Loss of FGF2 in FGF2-/- mice results in a 3-day delay in wound healing and at day 11 after wounding the mean wound

diameter was twice that of controls, the scab thickness 30% greater, and the percentage of reepithelialization was only 60% (ORTEGA *et al.* 1998). FGF7 (Keratinocyte Growth Factor) is the only FGF whose action is restricted to a single cell type, epithelial cells, in the adult mouse, and is thought to act in a paracrine manner as it is expressed in mesenchymal cells. FGFR2-IIIb, the only known high affinity receptor for FGF7 is expressed in keratinocytes of the epidermis and hair follicles. FGF7 is expressed weakly in mouse and human skin, but upon skin injury there is a striking induction in dermal fibroblasts (MARCHESE *et al.* 1995; WERNER *et al.* 1992). FGF7 mutant mice do not appear to have any defects, even in wound healing. It is likely then that several ligands function cooperatively and redundantly to orchestrate wound repair. FGF22, which is homologous to FGF7 and FGF10 is also thought to be used in wound repair (BEYER *et al.* 2003). It is expressed especially highly in hyperthickened wound epidermis and is therefore positioned well to be used in later stages after injury when a strongly hyperthickened epidermis is covering the wound, and this is the time *FGF22* transcripts are found to be upregulated.

Angiogenesis, or new blood vessel growth, is essential to the repair process of wound healing because it allows for the delivery of nutrients and oxygen to support the energy-consuming process of tissue remodeling. FGF1, FGF2, FGF8b and FGF4 are all potent pro-angiogenic growth factors which stimulate new vessel formation and vessel maturation by driving endothelial cell proliferation, promoting extracellular matrix degradation, altering intercellular adhesion and affecting communication through cadherins junctions, gap junctions and integrin expression (PRESTA *et al.* 2005).

The adult liver is also dependent on FGF signaling. FGF4 is found in mature hepatocytes and its disruption leads to abnormal liver function including depleted gallbladders, elevated bile acid pool and elevated excretion of bile acids (COUMOUL and DENG 2003a; YU *et al.* 2000).

FGF23 is important for the regulation of serum phosphate levels in adult animals (BAI *et al.* 2004). The mechanism of FGF23 regulation is explored in detail in the next section, but it is essential for proper kidney function and mutations in FGF23 lead to renal phosphate wasting disorders.

Regulation of FGF Signaling

Uncontrolled FGF signaling can lead to developmental abnormalities and disease, necessitating multiple layers of regulatory mechanisms to keep its activity in check.

Proteolytic Cleavage

One of the most notable stories of FGF regulation and its ties to disease comes from FGF23, which controls phosphate homeostasis in humans. FGF23 was originally identified as the mutated gene in patients with autosomal dominant hypophosphatemic rickets (ADHR), the phosphate wasting disorder and also as the causative factor of tumor-induced osteomalacia. In ADHR reabsorption of phosphate by the kidneys is impaired and leads to rickets, which is a softening or weakening of the bones. Osteomalacia is also a softening of bone tissue and it has been found that some tumors can induce this condition by causing an overproduction of FGF23.

FGF23 is produced as a full-length protein of 251 amino acids. It signals by binding to a complex of FGFR and Klotho, an obligate co-receptor. Cleavage of FGF23 at the ^{176}RXXR^{179} site, located at the boundary between the FGF core homology domain and the 72-residue C-terminal tail, results in inactivation of FGF23 and the production of a non-functional N-terminal fragment. Mutations in the proteolytic cleavage site lead to an accumulation of bioactive FGF23 and increased phosphate excretion resulting in hypophosphatemia.

Recent work has shown that the C-terminus of FGF23 is responsible for binding the full-length ligand to the FGFR/Klotho complex (GOETZ *et al.* 2010). The cleaved C-terminus can also compete for binding to further regulate signaling by FGF23. A minimal FGFR-Klotho binding epitope was identified which includes residues 180 to 200 of FGF23. FGF23$^{28\text{-}200}$, which is missing the last 51 C-terminal amino acids, still retains function and FGF23$^{180\text{-}200}$ is able to inhibit binding of FGF23 to the binary FGFR-Klotho complexes. Goetz and colleagues were also able to show that there was therapeutic potential to the inhibitory C-terminal domain. Treatment of *Hyp* mice, a mouse model of human X-linked hypophosphatemia, with the C-terminal FGF23$^{180\text{-}251}$ resulted in a decrease in phosphate excretion by the kidney.

Alternative splicing

An important aspect of FGF signaling in vertebrates is that many FGFR isoforms are generated by alternative splicing of *fgfr* transcripts (ESWARAKUMAR *et al.* 2005). Alternative splicing of the *fgfr* transcript gives rise to more than 48 major receptor isoforms. Alternative splicing in the D3 Ig-like extracellular domain exists in FGFR1,

FGFR2 and FGFR3 but not FGFR4. For FGFR, exon 7 encodes the N-terminal half of D3 (‘a’) and exon 8 and 9 alternatively encode for the C-terminal half of D3 (‘b’ or ‘c’). This is diagramed in Figure 4. Splicing in the D3 domain profoundly alters ligand-binding specificity (MIKI *et al.* 1992; YAYON *et al.* 1992). For instance, FGFR2b binds FGF7 and FGF10 but not FGF2. FGFR2c will bind FGF2 and FGF18, but not FGF7 and FGF10. Different isoforms are often expressed in different tissues: while the FGFR2b isoform is exclusively expressed in epithelial cells, the FGFR2c isoform is expressed exclusively in mesenchymal cells (ORR-URTREGER *et al.* 1993). The tissue-specificity allows communication between epithelial and mesenchymal tissues during development through the use of different FGF ligands. Alternative splicing has not been found for FGFR in invertebrates.

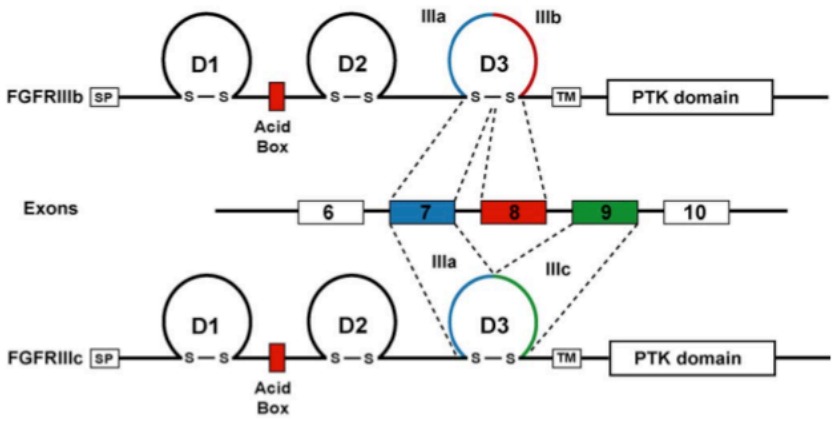

Figure 5 | Alternative Splicing of FGFR. Figure from (ESWARAKUMAR *et al.* 2005). The two forms of FGFR3 are generated by alternative splicing of exon 8 and 9. The C-terminal half of D3 is encoded by exon 8 to generate the ‘b’ isoform while exon 9 is used to generate the ‘c’ form.

Sproutys

FGF signaling is regulated by Sprouty proteins. Sprouty was discovered in a screen for mutations that affect tracheal branching in *Drosophila* (HACOHEN *et al.* 1998). In wild type embryos, FGF signaling through Bnl/Btl guides outgrowth of primary tracheal branches and induces cells closest to the FGF signaling center to form secondary branches. In *sprouty* (*spry)* null mutants, the FGF pathway is overactive and produces ectopic secondary branches that are induced in cells farther from the FGF signaling source. Overexpression of *spry* during primary branch outgrowth causes the opposite phenotype, inhibiting the FGF pathway and blocking all secondary branching. Therefore, Spry antagonizes FGF signaling in the trachea. Additionally, FGF signaling induces the expression of *spry*, and thereby regulates the expression of its own antagonist. This allows the embryo to regulate the range over which FGF signaling is active. Spry also regulates EGF signaling and other RTK signaling pathways.

A family of Spry homologs was identified in vertebrates and the mechanism of negatively regulating the FGF signaling pathway was found to be conserved (MINOWADA *et al.* 1999).

Heparan Sulfate Proteoglycans

The important role of Heparan Sulfate Proteoglycans (HSPGs) in developmental signaling in general, and FGF signaling in particular, is becoming more understood and appreciated. Heparin, an oversulfated intracellular variant of the ubiquitous Heparan Sulfate, was first discovered in 1916 for its ability to inhibit coagulation (WHITELOCK and IOZZO 2005). It was further developed over the next 20 years and was first tested in patients as an

anticoagulant drug in the mid 1930's. It was named 'hepa'rin/'hepa'ran after a common and abundant source of the molecule, hepatic tissue, from which it was first isolated and studied. Heparan sulfate was originally found as an impurity in heparin preparations. It is a complex and highly active biopolymer, which is synthesized as an alternating copolymer of hexuronic acid and glucosamine and modified at various positions with sulfate.

As RTKs, FGFRs can only activate themselves when they are brought together as dimers, and this dimerization requires the ligand to be either a multimer itself or large enough to bind two receptors at once. FGF ligands are small; as a result, they multimerize themselves by binding to HSPGs, either on the cell surface or in the extracellular matrix, and in this way can cross-link adjacent FGF receptors to produce the autophosphorylation of their cytosolic tails. The role of HSPGs in FGF signaling *in vivo* has not been studied in great detail (especially in *Drosophila*). HSPGs consist of a core protein with glycosaminoglycan (GAG) chains attached. All heparan GAG chains undergo some N-deacetylation/N-sulfation and O-sulfation in the Golgi and are therefore referred to as heparan sulfate (HS). Sulfation is responsible for most of the structural diversity of HS chains, and this heterogeneity could lead to binding specificity.

There are three types of proteoglycans: syndecans, glypicans, and perlecans (LIN and PERRIMON 2002). Syndecans and glypicans are both integral membrane proteins, and while glypicans have exclusively HS chains, syndecans have both HS and chondroitin sulfate attachments. Perlecans have only HS chains, are not membrane-attached, and are secreted into the extracellular matrix. In *Drosophila*, one syndecan gene, two glypican

genes, and a perlecan gene have been identified (LANDER and SELLECK 2000; PERRIMON and BERNFIELD 2000). *Drosophila* homologs have also been identified for genes encoding enzymes required for HS modifications: *sugarless, sulfateless* and *tout velu*, which encode homologs of UDP-D-glucose dehydrogenase, HS N-deacetylase/N-sulfotransferase and HS polymerase EXT-1, respectively (LIN and PERRIMON 2002). The *Drosophila* perlecan is encoded by the *terribly reduced optic lobe (trol*) gene. Mutations in *trol* reduce FGF signaling activity and cause cell-cycle arrest of neuroblasts in the larval brain. This phenotype can be rescued by adding human FGF2 to mutant brains in culture (HÄCKER *et al.* 2005).

Sulfation of HS has been shown to be important for FGF signaling. Inhibiting sulfate modification abolishes FGF signaling in cell culture (RAPRAEGER *et al.* 1991). Subsequently, 6-O-sulfation of N-acetylglucosamine was shown to be the key feature in HS that determines efficient binding of FGF1 and FGF2 to the FGF receptor (PELLEGRINI *et al.* 2000). A *Drosophila* 6-O-sulfotransferase (6-OST) was shown to be involved in tracheal development when RNAi-mediated inhibition of 6-OST activity disrupted tracheal development and showed similarity to Breathless mutants (KAMIMURA *et al.* 2001).

HSPGs are also important for Wnt signaling and they are thought to maintain the solubility of hydrophobic Wnt proteins by preventing their aggregation in the extracellular environment, thus stabilizing their signaling activity (FUERER *et al.* 2010).

FGF Signaling in Cancer

A screen looking at somatic mutations in 210 different human cancers found that mutations in components of the FGF signaling pathway were more common than mutations in any other signaling family (GREENMAN *et al.* 2007). The relationship between FGF signaling and cancer is complex as FGF signaling can drive tumorigenesis but in different contexts it can also mediate tumor protective functions. Due to the fact that FGF signaling regulates many cell behaviors including proliferation, differentiation, migration and survival, it is easy to see how uncontrolled FGF signaling can lead to cancerous cell behaviors.

Bladder cancer has the most established link to FGFR mutations. Approximately 50% of bladder cancers have somatic mutations in the *FGFR3*-coding sequence (CAPPELLEN *et al.* 1999). Most of the mutations in FGFR3 occur at a single position in the extracellular domain (S249C). This mutation leads to the formation of a new intermolecular cysteine disulphide bridge, leading to constitutive dimerization and activation of the receptor (TURNER and GROSE 2010). FGFR3 mutations are also linked to cervical cancers, multiple myeloma, prostate cancer, spermatocytic seminomas, oral squamous carcinomas, and in seborrhoeic keratosis (a benign wart-like growth that does not progress to malignancy). FGFR2 mutations have been described in endometrial carcinomas where the cancer cells are highly sensitive to FGFR inhibitors reflecting an oncogenic addition to the mutant-activated FGFR.

Amplification of the chromosomal region 8p11-12, which is the genomic location of *FGFR1* is one of the most common focal amplifications in breast cancer, predominantly in oestrogen receptor-positive cancers. Overexpression of wild-type FGFR1 occurs in cancer,

yet it is unclear whether the higher levels of FGFR1 lead to tumors with an aberrant response to paracrine FGF ligands, like FGF2, or whether at higher levels of FGFR1 expression ligand-independent signaling takes over.

Cancer can also result in the upregulation of FGF ligands while the switch of FGFR splicing can lead to the expression of a different receptor isoform. FGF2 and FGF6 are upregulated in prostate cancer and FGFR1-IIIc is upregulated while FGFR1-IIIb is downregulated. Loss of negative regulators of FGF signaling, like Sprouty2 and Sprouty2 and SEF can also increase FGF signaling in prostate cancer.

The mitogenic, cell-proliferation effects of FGF signaling can be enhanced by pro-survival signaling. FGF signaling has the potential in some cell types to activate anti-apoptotic pathways through the activation of the P13K-AT or STAT signaling pathways. This cell survival effect has been linked to resistance to chemotherapy. In addition to effects on proliferation and survival, FGF signaling can promote cell migration, leading to tumor invasion, and epithelial-to-mesenchymal transition (EMT), which is important in cancer cell metastasis. FGFR1 is thought to mediate these effects in breast cancer model mice by upregulation of the FGF target and pro-EMT gene *Sox9*.

There is unequivocal evidence from mouse models for a tumor suppressive role of FGFR2 in some cellular contexts. Mice lacking FGFR2-IIIB in keratinocytes are sensitive to carcinogenic insults to their skin. Studies on a rat model of prostrate cancer showed that when non-malignant cells expressing FGFR2-IIIb were mixed with cancer cells they formed non-malignant tumors. In bladder cell lines, expression of FGFR-IIIb blocks

proliferation, and it is also downregulated on progression in bladder cancers, prostate cancer, and salivary adenocarcinomas. How the tumor-suppressive function of FGFR2-IIIb is mediated is not clear. One proposal is that FGF signaling may induce cytoprotective pathways in epithelial cells and help them to maintain genomic stability following carcinogenic insult.

Therapeutic efforts are currently focused on FGFR specific tyrosine kinase inhibitors (TKIs). One complication that has already developed from using TKIs is that pan-inhibition of all FGFR leads to hyperphosphataemia-mediated tissue calcification through blockage of FGF23 signaling. More specific therapeutic antibodies have been made to reduce toxicity. Antibodies targeting FGFR3 have been shown to have an anti-proliferative effect on bladder cancer cells. A third approach has been to develop FGF ligand traps by making soluble fusion proteins that consist of the FGFR extracellular domain fused to the Fc domain of IgG1.

Summary

The field of FGF signaling has grown to 34,410 papers on PubMed as of September 2010. So many exceptions have now been found to characteristics that were once thought of as rules that there are few remaining "defining" features that fit all known FGF interactions, but many detailed mechanisms are still not understood. The importance of FGF signaling in development and medicine has now been firmly established and future research directions are now centered on carefully working out the details of mechanisms, cross-talk networks, evolutionary history and therapeutic applications.

Chapter 2

INTRODUCTION TO PROTEOLYTIC PROCESSING IN DEVELOPMENTAL SIGNALING

Proteolysis is a powerful mechanism for the posttranslational control of cell signaling pathways. Some proteolytic events like the action of the proteosome result in the wholesale destruction of target proteins, but many involve only "limited proteolysis" at one or a few specific cleavage sites in the substrate. These specific cleavages can lead to activation or inactivation of proteins and often involve conformational changes or separation of interacting domains. Unlike phosphorylation, proteolytic cleavage is irreversible, but sometimes an activating cleavage is followed by a secondary cleavage that serves to inactivate the protein. Due to the dramatic effect proteases have on protein function, complex regulatory mechanisms are also in place to control their substrate specificity, catalytic activity, localization, and timing of activity (LEMOSY 2006).

Although my thesis work was the first description of proteolytic activity on FGF ligands in *Drosophila*, cleavage is a common mechanism for regulation of developmental signaling and in this introductory chapter I will introduce other signaling families and organisms where the process has been described in detail. The systems I will highlight are TGF-β (including Dpp), Notch/Delta, EGF, the Cysteine Knot Growth Factor family (Spätzle), and FGF23.

Cleavage of TGF-β ligands by proprotein convertases

TGF-β ligands, including BMPs in vertebrates and their homolog Decapentaplegic (Dpp) in *Drosophila* are all initially produced as inactive proproteins precursors. The proproteins are subsequently cleaved by proprotein convertase enzymes of the Furin family to generate the active ligand (AONO *et al.* 1995; CUI *et al.* 1998). Furin, the first member of this family to be characterized, is a membrane-associated, calcium-dependent serine endoprotease that proteolytically activates at the C-terminal side of a consensus site consisting of basic Arg-X-X-Arg residues (MOLLOY *et al.* 1992).

BMP signaling is required for proper development and one of its earliest and best-documented roles is in the establishment of the dorsoventral axis, reviewed in (GRAFF 1997). In *Xenopus* embryos, expression of BMP-4 is restricted to cells on the ventral side of gastrula-stage embryos, where it specifies ventral mesoderm. Also, BMP is necessary for the induction of epidermal fate at the expense of neural tissue. BMPs are powerful molecules able to induce ectopic bone in non-bony tissue. It is therefore imperative that BMP activity be tightly controlled.

It was first shown that BMPs were cleaved by proprotein convertases when an inhibitor engineered to block endogenous proprotein convertase activity was injected into *Xenopus* embryos and the result phenocopied the effect of blocking BMP activity (i.e. dorsalizing ventral marginal zone cells) (CONSTAM and ROBERTSON 1999; CUI *et al.* 1998). Further analysis showed that BMP4 is cleaved sequentially at two sites: first at a site adjacent to the mature ligand domain (S1 site) and then at an upstream site in the prodomain (S2 site) (CUI *et al.* 1998). Mutant forms of BMP4 lacking this second site

cleavage revealed that the cleaved prodomain remains associated with the mature ligand and the complex is targeted for rapid degradation in the absence of the secondary cleavage (DEGNIN *et al.* 2004). This mechanism of regulation is not unprecedented. The propeptide of Furin itself is excised at a Furin site but remains noncovalently associated and functions as an autoinhibitor of the active zymogen until a secondary cleavage releases the active enzyme (ANDERSON *et al.* 1997). BMP-4 (and Dpp) can function as either short-range or long-range signals depending on the tissue in which they are expressed (NEUMANN and COHEN 1997), and alternative cleavage strategies could mediate this decision. Cleavage at both the S1 and S2 sites generates a stable ligand that possesses long-range signaling properties, whereas cleavage at the S1 site alone creates a ligand that is rapidly degraded and can only signal to adjacent cells (DEGNIN *et al.* 2004). The mechanism for the tissue-specificity is thought to be mediated by Furin family members that are localized to distinct post-trans Golgi network compartments with different levels of acidity (DEGNIN *et al.* 2004).

Processing of Decapentaplegic *and* Glass bottom boat *in* Drosophila

Drosophila proteins Dpp and Glass Bottom Boat (Gbb), like their vertebrate BMP homologs, are produced as inactive preproproteins and cleaved by Furin1 and Furin2 to release the mature, active protein (KÜNNAPUU *et al.* 2009). Dpp is the ortholog of vertebrate BMP-2/4, but is cleaved in a different manner, indicating that even though they are functional orthologs, Furin-cleavage sites are tolerant to mutations acquired through evolution. Dpp is produced as two molecular forms in both cell culture and the embryo. The production of these forms requires a multi-step process using the Furin site II and

Furin site III to make the large form (Dpp26) and Furin site II and Furin site I to make the small form (Dpp23) (KÜNNAPUU *et al.* 2009). Cleavage at the Furin site II is critical and sufficient for long-range Dpp signaling in wing development (KÜNNAPUU *et al.* 2009). Recent studies have revealed that tissue-specific differential cleavage is important for the activity of Dpp during *Drosophila* development. Dpp is processed in a tissue-dependent manner and different cleavage products are required to provide sufficient function for wing and leg versus gut development (KÜNNAPUU *et al.* 2009; WHARTON and DERYNCK 2009).

Glass bottom boat (Gbb), the homolog of BMPs 5, 6, 7, and 8, is also subject to Furin processing in a tissue-dependent manner. Unlike Dpp, however, one of the two resulting forms has a long N-terminal extension, which is capable of signaling. Receptor binding of an incompletely processed ligand is not unprecedented, as the TGF-β Nodal is secreted as a full-length, uncleaved precursor in the mouse embryo where it binds the activin receptor to maintain expression of proprotein convertases) (BEN-HAIM *et al.* 2006). The Furin-like proteases then act at the cell surface to cleave nodal.

Notch undergoes multiple cleavage events

The family of Notch receptors mediates binary cell fate decisions through short-range signaling during development across the metazoa. Through binding to Delta, Serrate, and Lag-2 ligands, Notch signaling participates in what is known as “lateral inhibition” where a field of cells have equal potential to adopt a certain fate over a secondary fate and once a

cell has made the decision it activates Notch in neighboring cells to suppress those cells from adopting the same cell fate (EHEBAUER *et al.* 2006). Processing of the Notch receptor is essential to the functional roles it plays in development.

Notch is produced as a single polypeptide but is then processed multiple times to mediate the presentation of the receptor and to facilitate signaling (Figure 1). Cleavage at a site designated S1 happens in the secretory pathway by a Furin-like protease within the trans-Golgi vesicles (BLAUMUELLER *et al.* 1997; LOGEAT *et al.* 1998). This cleavage produces two fragments, one of which contains most of the extracellular domain and the other contains the remaining extracellular domain and the membrane-tethered intracellular region. These two polypeptide fragments remain non-covalently associated in a Ca^{2+} manner (RAND *et al.* 2000). Upon ligand binding, Notch is further processed by two different proteases at sites S2 and S3.

Cleavage at S2 is catalyzed by an ADAM/TACE/Kuzbanian family metalloprotease and it releases the extracellular domain from the receptor, leaving behind the membrane-tethered intracellular domain (BROU *et al.* 2000; LIEBER *et al.* 2002; MUMM *et al.* 2000). Cleavage at S3 takes place inside the nicastrin-transmembrane helix and is catalyzed by the γ–secretase activity of the presenilin-Aph1-Pen2 protein complex (FORTINI 2002). This last cleavage event releases the intracellular domain, which then enters the nucleus and interacts with members of the CSL family of transcription factors to turn on downstream genes.

The Delta transmembrane ligand also undergoes three proteolytic cleavages induced by Notch and one of these cleavages is dependent on the ADAM metalloprotease Kuzbanian (BLAND *et al.* 2003).

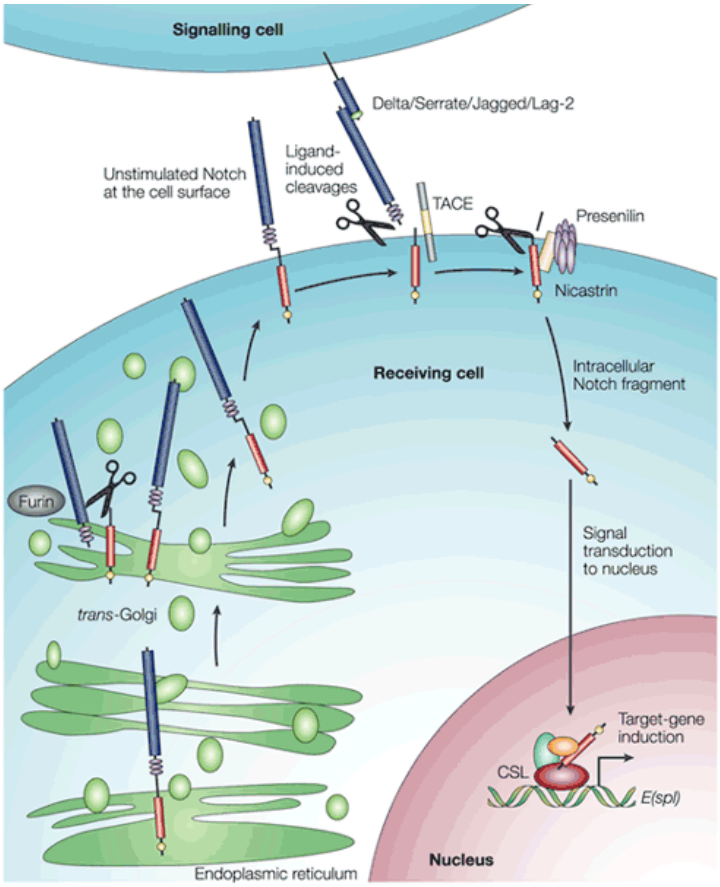

Figure 1 | Processing of Notch. From (FORTINI 2002). Notch is synthesized as a 300-kDa precursor that undergoes three distinct cleavages. In the *trans*-Golgi vesicles the Notch precursor is cleaved by a Furin-like convertase to generate separate extracellular and transmembrane/intracellular domains. These segments are joined by a noncovalent attachment to create a heterodimeric form of Notch, which is the main form detected at

the cell surface. Notch binding of ligands of the Delta/Serrate/Jagged family results in ectodomain removal due to extracellular cleavage mediated by the TACE/ADAM metalloproteinases. The membrane-anchored carboxy-terminal fragment that is left after this cleavage event is subsequently processed by the γ-secretase complex, which contains presenilin. This intramembrane cleavage liberates the Notch intracellular domain, which translocates to the nucleus to regulate the expression of target genes in association with other CSL family nuclear factors.

Cleavage of EGF ligand Spitz by Rhomboid

The EGF signaling pathway plays multiple roles during development: the establishment of ventral ectodermal fates, differentiation of midline glial cells, Malpighian tubule development, germ-band retraction, head development, and proliferation of imaginal disc development. In *Drosophila*, the Spitz (Spi) protein is the ligand for the DER (Drosophila Egf Receptor). Spi is cleaved in its transmembrane domain to release a secreted form (sSpi) that can bind to DER (LEE *et al.* 2001; URBAN *et al.* 2001). The protease that cleaves Spi is Rhomboid and although it was known for many years that *spi* and *rhomboid* had a genetic interaction, the description of the processing took ten years to figure out.

In 1992, Rutledge et al. gave a phenotypic and molecular description of Spi showing that it is most similar to TGF-α with a transmembrane domain and a potential cleavage site of a dibasic Arg-Lys between the EGF domain and transmembrane domain (RUTLEDGE *et al.*

1992). The isolation of cDNAs allowed them to say the *spi* gene is capable of producing a 26kB protein (unmodified). Then, in 1994, Matthew Freeman used eye disc photoreceptor mosaic genetics to suggest that Spi had a diffusible form (FREEMAN 1994). In 1995 Schweitzer and colleagues transfected S2 cells with full length Spi and a short form of Spi containing all amino acids up until the putative cleavage domain. This short form was able to activate DER in S2 cells (shown with an antibody against phosphotyrosine) and when overexpressed in embryos using Kr-GAL4, it caused a ventralization phenotype (SCHWEITZER *et al.* 1995).

Conclusive evidence came from the Freeman lab in 2001 by Lee et al. They show that Star is required to transport Spi from the ER to the Golgi and Rhomboid is localized in the Golgi, where it promotes cleavage of Spi (LEE *et al.* 2001). Co-transfection of Rhomboid and Star with Spi causes relocation of Spi to the Golgi and plasma membrane (there is no Spi at the surface when Star is not present). Sequential degylcosylation revealed Spi is glycoslyated in the ER (N-linked sugars) and the Golgi (O-linked sugars). A series of deletion and chimera proteins demonstrated that the lumenal domain of Spi is important for relocalization of Spi. Finally, by Western blot, it was determined that the transmembrane domain of Spi is essential for cleavage.

Concurrently, also from the Freeman lab, Urban et al. showed the mechanism of Rhomboid cleavage of Spi. Rhomboid is a novel serine protease that cleaves Spi in its transmembrane domain (URBAN *et al.* 2001). To determine the cleavage domain, mutations were made in Rhomboid at the eleven non-glycine conserved residues. Only six mutations affected Rhomboid's ability to cleave Spi. The sequence between two of the conserved amino acids

is a motif shared with other serine proteases, and the amino acids making up the predicted catalytic triad are all essential. To show that Spi is cleaved in an equivalent position in its transmembrane domain to that of the Rhomboid active site, truncated forms of Spi were compared to Rhomboid-mediated cleaved Spi. Rhomboid was tagged with a KDEL motif to retain it in the ER, so Spi would be cleaved before being glycosylated (which would change the molecular weight). Cleaved Spi was larger than a truncation just before the transmembrane domain at residue 139 but smaller than a truncation 2/3 of the way in the transmembrane domain at residue 149. The resolution ability of the gel assay was about 5 residues, so the cleavage site was determined to be close to residue 144, on the side of the lumenal face. A series of class specific protease inhibitors were used to show that Rhomboid was indeed a serine protease, sensitive to TPCK and DCI inhibitors. Rhomboids are very well conserved proteins and a human Rhomboid can also support Spi cleavage.

Spätzle and the formation of the dorsal-ventral axis in *Drosophila*

Cleavage of signalling ligands plays an important role in the well-characterized process of dorsal-ventral axis specification in the *Drosophila* oocyte (Figure 2). Different territories along the D/V embryonic axis are specified by graded nuclear localization of the Dorsal transcription factor. The translocation of Dorsal to the nucleus is a consequence of the activation of the Toll receptor by the ligand Spätzle. Although the Toll receptor is uniformly distributed, the Spätzle ligand is only modified into its active form on the ventral side of the embryo where a proteolytic cascade is activated by Pipe.

Pipe is inhibited on the dorsal side of the embryo by Torpedo signalling activated by the Gurken protein. The Gurken protein is synthesized from *gurken* mRNA transcripts, which are produced by the nucleus after it has travelled to the dorsal-anterior corner of the oocyte.

Clearly, the correct timing and location of activation of the Spätzle ligand is essential for a normal D/V axis to develop. The Spätzle ligand has a C-terminal cysteine knot present in many growth factors and only the C-terminal 106 amino acids is required to activate the Toll receptor. Spätzle is activated when cleaved away from an unstructured, inhibitory N-terminal domain by the Easter protease (DELOTTO and DELOTTO 1998; MORISATO and ANDERSON 1994; WEBER *et al.* 2003). Easter is a member of the trypsin family of serine proteases (CHASAN and ANDERSON 1989). The cleavage reaction is also hypothesized to produce a diffusible inhibitor of Spätzle, which contributes to the shaping of the Dorsal gradient (MORISATO 2001). This inhibitor may be the N-terminal domain of Spätzle. The detailed understanding of this process is technically difficult because it takes place in the perivitelline space and the vitelline membrane is a barrier to detection reagents and is usually removed during fixation of *Drosophila* embryos. Spätzle is also active in the Toll-mediate immune response in flies. Possibly studies on the method of Spätzle processing during the immune response can bypass the technical barriers of the oocyte and provide insight into the general mechanism necessary for proper production of active Spätzle form.

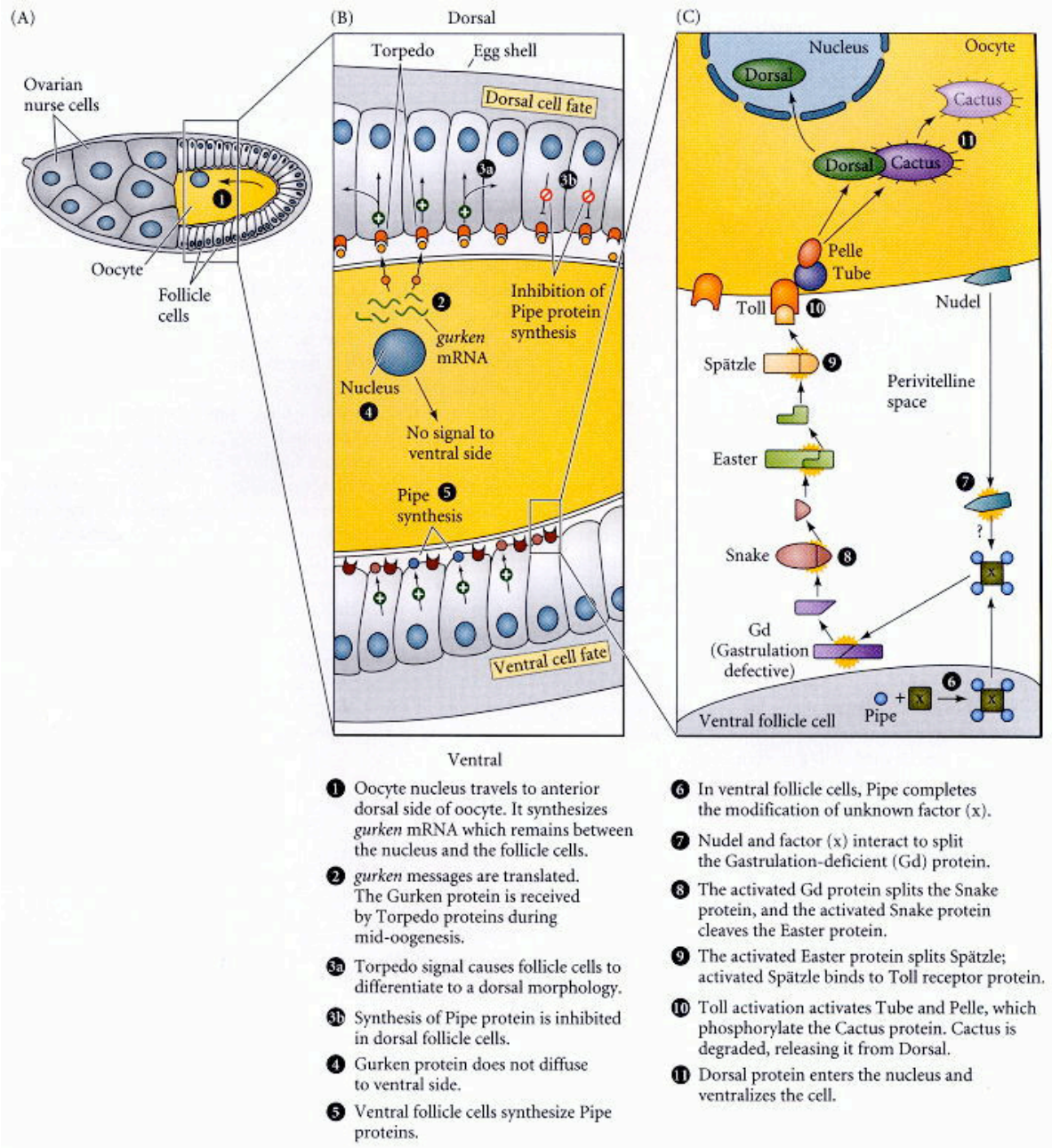

Figure 2 | Proteolysis in generating dorsal-ventral polarity in *Drosophila*. From (GILBERT 2006). (A) The oocyte nucleus travels anteriorly and secretes *gurken* mRNA which is translated into Gurken protein. Gurken protein causes follicle cells adopt a dorsal fate by inhibiting the production of Pipe. The production of Pipe in ventral

follicle cells causes the secretion of an unknown factor which works with Nudel to split Gd. Gd then cleaves snake which then goes on to cleave Easter. Easter cleaves Spätzle and allows it to bind Toll receptor.

Processing of FGF ligands

Regulation of ligand activity by cleavage has only been described for one member of the FGF family, FGF23. The details of this regulation were detailed in Chapter 1 under the "Regulation/Proteolytic Cleavage" section. From the many examples of regulatory cleavage events described here for other signaling molecules, it should be clear that this is a very common mechanism of regulating cell signaling in development. We have found that two FGF ligands in *Drosophila* are cleaved and our evidence is detailed in Chapter 3.

Chapter 3

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ANALYSIS OF THISBE AND PYRAMUS FUNCTIONAL DOMAINS REVEALS EVIDENCE FOR CLEAVAGE OF *DROSOPHILA* FGFS

Abstract

As important regulators of developmental and adult processes in metazoans, Fibroblast Growth Factor (FGF) proteins are potent signaling molecules whose activities must be tightly regulated. FGFs are known to play diverse roles in many processes, including mesoderm induction, branching morphogenesis, organ formation, wound healing and malignant transformation; yet much more remains to be learned about the mechanisms of regulation used to control FGF activity. In this work, we conducted an analysis of the functional domains of two *Drosophila* proteins, Thisbe (Ths) and Pyramus (Pyr), which share homology with the FGF8 subfamily of ligands in vertebrates. Ths and Pyr proteins are secreted from *Drosophila* Schneider cells (S2) as smaller N-terminal fragments presumably as a result of intracellular proteolytic cleavage. Cleaved forms of Ths and Pyr can be detected in embryonic extracts as well. The FGF-domain is contained within the secreted ligand portion, and this domain alone is capable of functioning in the embryo when ectopically expressed. Through targeted ectopic expression experiments in which we assay the ability of full-length, truncated, and chimeric proteins to support cell

differentiation, we find evidence that (1) the C-terminal domain of Pyr is retained inside the cell and does not seem to be required for receptor activation and (2) the C-terminal domain of Ths is secreted and, while also not required for receptor activation, this domain does play a role in limiting the activity of Ths when present. We propose that differential protein processing may account for the previously observed inequalities in signaling capabilities between Ths and Pyr. While the regulatory mechanisms are likely complex, studies such as ours conducted in a tractable model system may be able to provide insights into how ligand processing regulates growth factor activity.

Background

Fibroblast Growth Factors (FGFs) comprise a large family of signaling molecules that are key regulators of developmental processes including mesoderm induction, gastrulation, cell migration, midbrain-hindbrain patterning, limb induction and bone formation (CIRUNA and ROSSANT 2001; CROSSLEY *et al.* 1996; NISWANDER *et al.* 1993; REIFERS *et al.* 1998; SHIANG *et al.* 1994; SLACK *et al.* 1987; THISSE and THISSE 2005). FGFs continue to function in adult tissue homeostasis and wound healing; when improperly activated they can also contribute to many human diseases and cancer (CHEN and DENG 2005; COUMOUL and DENG 2003b; ESWARAKUMAR *et al.* 2005; THISSE and THISSE 2005). Most of the 24 known FGF ligands in vertebrates are small proteins with a molecular mass of 17-34 kD, whereas the three known *Drosophila* FGF ligands are all predicted to be much larger proteins with molecular masses of approximately 80 kD (DRAPER *et al.* 2003; ORNITZ and ITOH 2001b). Vertebrate FGFs and *Drosophila* FGFs share homology within their FGF

domains, but *Drosophila* FGFs have an additional long, low-complexity sequence of unknown function.

The FGF ligands in *Drosophila* are Branchless (Bnl), Thisbe (Ths), and Pyramus (Pyr), and they bind to FGF receptors (FGFR), which are receptor tyrosine kinases (RTKs). FGF signaling is used pervasively throughout development. Bnl-mediated activation of the Breathless (Btl) receptor controls branching of the developing trachea (SUTHERLAND *et al.* 1996), while Ths and Pyr activate the Heartless (Htl) receptor to control movement of the mesoderm cells (GRYZIK and MÜLLER 2004b; KADAM *et al.* 2009; MCMAHON *et al.* 2008; STATHOPOULOS *et al.* 2004; WILSON *et al.* 2005), pericardial cell specification (GRYZIK and MÜLLER 2004b; KADAM *et al.* 2009; KLINGSEISEN *et al.* 2009a; STATHOPOULOS *et al.* 2004), and caudal visceral mesoderm migration (MANDAL *et al.* 2004; SHISHIDO *et al.* 1997). Pyr and Ths ligands also function later in development within the nervous system to control glial cell proliferation, migration and axonal wrapping (FRANZDÓTTIR *et al.* 2009). Ths and Pyr are thought to share one receptor, which makes *Drosophila* an ideal model to study FGF signaling specificity and differential regulation. Initial work on the individual functions of Ths and Pyr in the embryo was recently described using genetic approaches, where it was found that although both ligands play a role in mesoderm spreading, Pyr is more important for pericardial cell specification (KADAM *et al.* 2009; KLINGSEISEN *et al.* 2009a).

In order to achieve a better understanding of how Ths and Pyr proteins are adapted to their particular roles, it is necessary to first understand the mechanism by which signaling with a

particular FGF ligand occurs, and the way this signaling is regulated. Signaling ligands can be intracellular, membrane-bound, or secreted, and are often modified and processed in many different ways. Understanding these basic properties of a signaling ligand provides important clues for any further mechanistic studies.

Proteolytic processing is a common regulatory mechanism of growth factors and other signaling pathways in both vertebrates and *Drosophila*. Examples from *Drosophila* include the EGF ligand Spitz (Spi), TGF-β ligands Decapentaplegic (Dpp) and Glass Bottom Boat (Gbb), Spätzle, Notch, and Delta. Spi is cleaved in its transmembrane domain to release a secreted form (sSpi) that can bind to the *Drosophila* EGF Receptor (DER) (LEE *et al.* 2001; URBAN *et al.* 2001). The Spätzle C-terminal cysteine knot is activated when cleaved away from an unstructured, inhibitory N-terminal domain (DELOTTO and DELOTTO 1998; MORISATO and ANDERSON 1994; WEBER *et al.* 2003). Dpp and Gbb, like their vertebrate BMP homologs, are produced as inactive preproproteins and cleaved by Furin1 and Furin2 to release the mature, active protein (KÜNNAPUU *et al.* 2009). Notch is produced as a single polypeptide but is then processed in the secretory pathway by a Furin-like protease within the Golgi to produce two fragments that remain non-covalently associated (BLAUMUELLER *et al.* 1997; LOGEAT *et al.* 1998; RAND *et al.* 2000). Lastly, Delta undergoes three proteolytic cleavages and one of these cleavages is dependent on the ADAM metalloprotease Kuzbanian (BLAND *et al.* 2003). Uncovering the proteolytic processing events of these growth factors and signaling molecules has led to a deeper understanding of their signaling mechanism and regulation.

Here we have found evidence for (1) the proteolytic cleavage of Ths and Pyr full-length precursor proteins and (2) the secretion of the FGF-domain-containing N-terminus. The role of proteolytic processing in FGF signaling is currently limited to one vertebrate FGF ligand, FGF23, which is part of a subgroup of endocrine FGFs. Full length FGF23 is 251 amino acids and is cleaved 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). These studies support the view that a delicate balance is necessary to control the level of secreted bioactive FGF proteins (BAI *et al.* 2003). We also show that after processing, Ths and Pyr are similar in size to their vertebrate homolog FGF8 (~30 kD) suggesting that studying regulation of FGF signaling in *Drosophila* could provide useful insights for the FGF field in general.

In addition to understanding the processing of Ths and Pyr, we sought to link the structural domains to the function of the ligands. From embryonic stage 10 to 11, the developing dorsal mesoderm requires activation of the Htl receptor to specify two *even-skipped* (*eve*) expressing progenitor cells, which give rise to three Eve-positive founder cells (BEIMAN *et al.* 1996). Two of these Eve-positive founders will become Eve-positive pericardial cells, and the third founder will give rise to dorsal somatic muscle (BUFF *et al.* 1998; CARMENA *et al.* 1998a; CARMENA *et al.* 1998b). When either Ths or Pyr are ectopically expressed throughout the neurogenic ectoderm using a 69B-GAL4 driver, the Eve-positive cell cluster increases from three cells to as many as 20 cells (KADAM *et al.* 2009; STATHOPOULOS *et al.*

2004). In this work we used these supernumerary Eve-positive cells as a functional readout of Ths and Pyr activity. By analyzing a series of truncation, deletion, and chimeric constructs, our results collectively suggest that the N-terminal FGF domain alone is sufficient to support function, but only when properly folded and secreted.

If the N-terminus alone is able to activate the receptor and allow downstream signaling, then what is the role of the long C-termini of Ths and Pyr? We addressed this question with another GAL4 driver, ZenKr-GAL4, which drives expression only in a subset of the dorsal ectoderm of the early embryo (i.e., zenVRE.Kr-GAL4 (FRASCH 1995)). Limiting the source of protein production to this restricted domain allowed us to assay differences in the range-of-action of different Ths and Pyr constructs. Our results suggest that the Ths C-terminus is inhibitory and the Pyr C-terminus is not. Collectively, these findings demonstrate that post translational processing is important for FGF signaling during embryonic development of *Drosophila* and suggest that processing of signaling ligands may be widespread.

Results

Comparison of predicted protein characteristics for Thisbe and Pyramus

A screen to uncover genes expressed during patterning the dorsal-ventral axis of *Drosophila*, identified expression of the *thisbe* gene (*ths*: previously called *Neu4*) in the neurogenic ectoderm (STATHOPOULOS *et al.* 2002). Results from additional genetic experiments were consistent with the hypothesis that Ths and Pyr are two FGF ligands for the Htl FGF receptor (GRYZIK and MÜLLER 2004b; STATHOPOULOS *et al.* 2004). To

understand the mechanism of FGF signaling through Htl on a molecular level, we characterized Ths and Pyr proteins by analyzing their functional domains. We first considered predictions about the size and homologous domains of Ths and Pyr.

The *ths* cDNA contains a 2,247 basepair open-reading frame and is predicted to encode a protein of 748 amino acids (aa) with a molecular weight of 82.2 kD. Thisbe is predicted to have an N-terminal signal peptide followed by a 122 aa FGF domain composed of 12 predicted β-strands separated by coiled-coil domains, which presumably support a trefoil structure like vertebrate FGFs. Beyond the FGF domain, however, the C-terminal domain of Ths exhibits only limited homology within deuterostomes, to other uncharacterized "immunoglobulin-like proteins" or proteins that are known to be highly glycosylated (data not shown). The Ths protein sequence also contains several dibasic and multi-basic motifs characteristic of the recognition site for Furin proteases (RHOLAM and FAHY 2009; SEIDAH and CHRÉTIEN 1999) and several predicted N-linked glycosylation sites (Fig. 1).

The *pyr* cDNA contains a 2,301 basepair open-reading frame and is predicted to encode a protein of 766 aa and ~87 kD. Pyr also has an N-terminal signal peptide followed by an FGF domain of 128 aa (Fig. 1). Ths and Pyr share 39% amino acid identity in the FGF core domain. C-terminal to the Pyr FGF domain, there are many repeats and regions of low complexity. From amino acids 399 to 426, Pyr has a string of hydrophobic amino acids

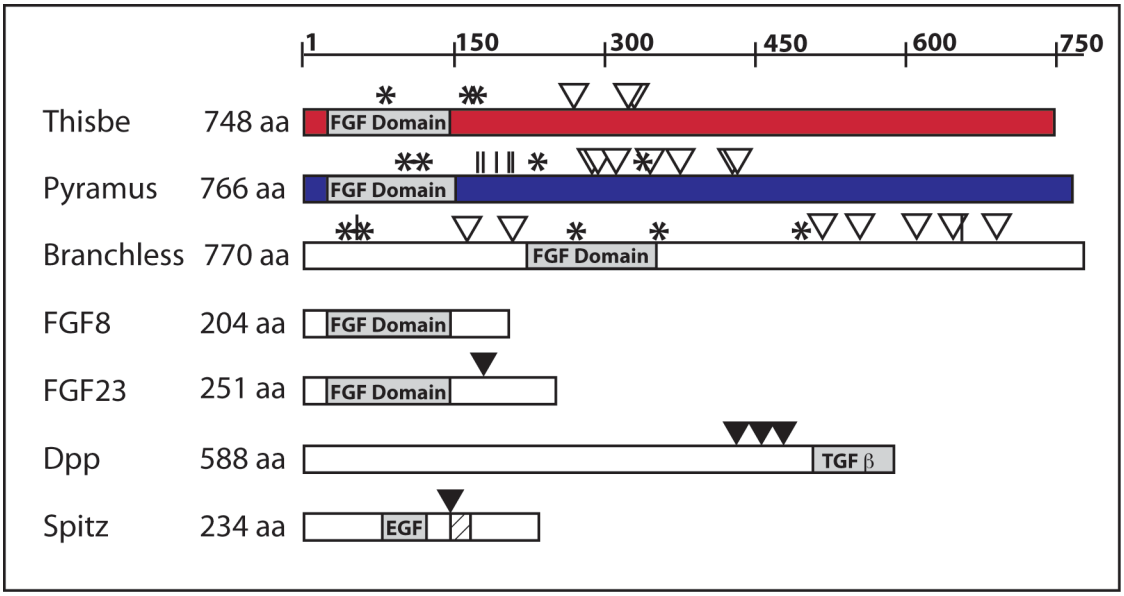

Figure 1 | Comparison of Ths and Pyr Proteins to other signaling ligands

thisbe and *pyramus* genes encode proteins of 748 and 766 amino acids (aa), respectively, making them far larger than their vertebrate homolog FGF8, which is 204 aa. Branchless, another FGF ligand in *Drosophila*, is also a relatively large protein of 770 aa. The C-terminus is cleaved from FGF23, the only FGF family member known to be cleaved (BENET-PAGÈS *et al.* 2004; FUKUMOTO 2005). Dpp is produced as a 588 aa precursor, but is cleaved to primarily the TFG-β-homologous domain alone (KÜNNAPUU *et al.* 2009; LEE *et al.* 2001; PANGANIBAN *et al.* 1990; SHIMMI *et al.* 2005). Spitz is processed within its transmembrane domain and, like Thisbe and Pyramus, binds to a RTK receptor to signal (LEE *et al.* 2001; URBAN *et al.* 2001). Known cleavage sites are marked with a black inverse triangle. In the *Drosophila* FGFs, potential cleavage sites consisting of multi-basic amino-acid motifs are marked with a white inverse triangle. Predicted N-glycosylation sites are marked with an asterisk and predicted O-glycosylation sites are marked with a vertical line.

that weakly qualifies as a potential transmembrane domain when assayed by topology prediction programs using the Kyte-Doolittle Scale (KYTE and DOOLITTLE 1982). Pyr also has sites of predicted N-linked and O-linked glycosylation and putative dibasic and multi-basic protease recognition sites (see symbols in Fig. 1).

Ths and Pyr are secreted from S2 cells and detectable as cleaved forms

To confirm the ability of the full-length *ths* and *pyr* cDNAs to support the production of ~80 kD proteins as predicted by their sequence, we expressed Ths and Pyr proteins *in vitro* using a rabbit reticulocyte transcription/translation system that incorporates S^{35}-labeled Methionine. Full-length proteins were detected at ~80 kD, as predicted (Fig. 2A).

We compared the size of Ths and Pyr proteins with other ligands (e.g., Bnl, FGF8, Dpp, Spi, and FGF23; see Fig. 1), and found Ths and Pyr to be much larger than FGF8 and closer in size to other cleaved growth factors in *Drosophila* like Dpp. Therefore, we hypothesized that Ths and Pyr may also be regulated by cleavage. Dpp (588aa) is activated by cleavage into much smaller molecules consisting primarily of the TGFβ-homologous domain (KÜNNAPUU *et al.* 2009; PANGANIBAN *et al.* 1990; SHIMMI *et al.* 2005). Spitz is an EGF ligand that, like Ths and Pyr, uses a high-affinity RTK receptor to signal. Spitz is cleaved within its transmembrane domain to release the EGF domain as a small, secreted ligand (LEE *et al.* 2001; URBAN *et al.* 2001). All FGFs in vertebrates, even the cleaved FGF23, are small molecules consisting mostly of the FGF domain alone. These comparisons led us to consider the hypothesis that Ths and Pyr may not signal as long, full-length proteins, but as small molecules consisting primarily of the FGF domain.

First, we sought to verify whether Ths and Pyr were indeed secreted proteins by transiently expressing *ths* and *pyr* from a metallothionine promoter in S2 cells, a cell line derived from *Drosophila* embryonic cells. Mutants deficient in both the *ths* and *pyr* genes (i.e. Def(2R)BSC25 STATHOPOULOS *et al.* 2004) phenocopy the *htl* mutant phenotype, but in the early *Drosophila* embryo Ths and Pyr proteins are expressed in the ectoderm while Htl is limited to the abutting mesoderm cells. Thus, for Ths and Pyr to influence the activity of the Htl FGFR in the mesoderm, our working hypothesis had been that the FGF ligands are secreted from the ectoderm to activate the FGFR present in the mesoderm. Consistent with this view, signal sequences are predicted at the N-terminus within the identified protein sequences (STATHOPOULOS *et al.* 2004). Nevertheless, we sought to examine secretion directly. In order to follow both the N- and C-termini separately, we constructed epitope-tagged constructs with a single hemagglutinin (HA) tag at the N-terminus after the signal peptide and a 6X Myc tag at the C-terminus (diagrams in Fig. 2B). UASt.HA-Ths-Myc and UASt.HA-Pyr-Myc plasmids were co-transfected into S2 cells along with the metallothionine-inducible Gal4 plasmid and ectopic expression of the tagged proteins was achieved by copper induction. Using anti-HA antibody, we were able to immunoprecipitate N-terminally tagged Ths and Pyr from the culture medium,

demonstrating directly that the proteins are indeed secreted (Fig. 2B, lanes 2 and 3). Instead of identifying secreted proteins at the predicted full-length molecular weights, we found the predominant secreted forms consisted of multiple bands running at ~35 kD for Ths and ~50 kD for Pyr (Fig. 2B, lane 2 and 3, respectively), indicating that the cleaved N-terminus of each protein is secreted.

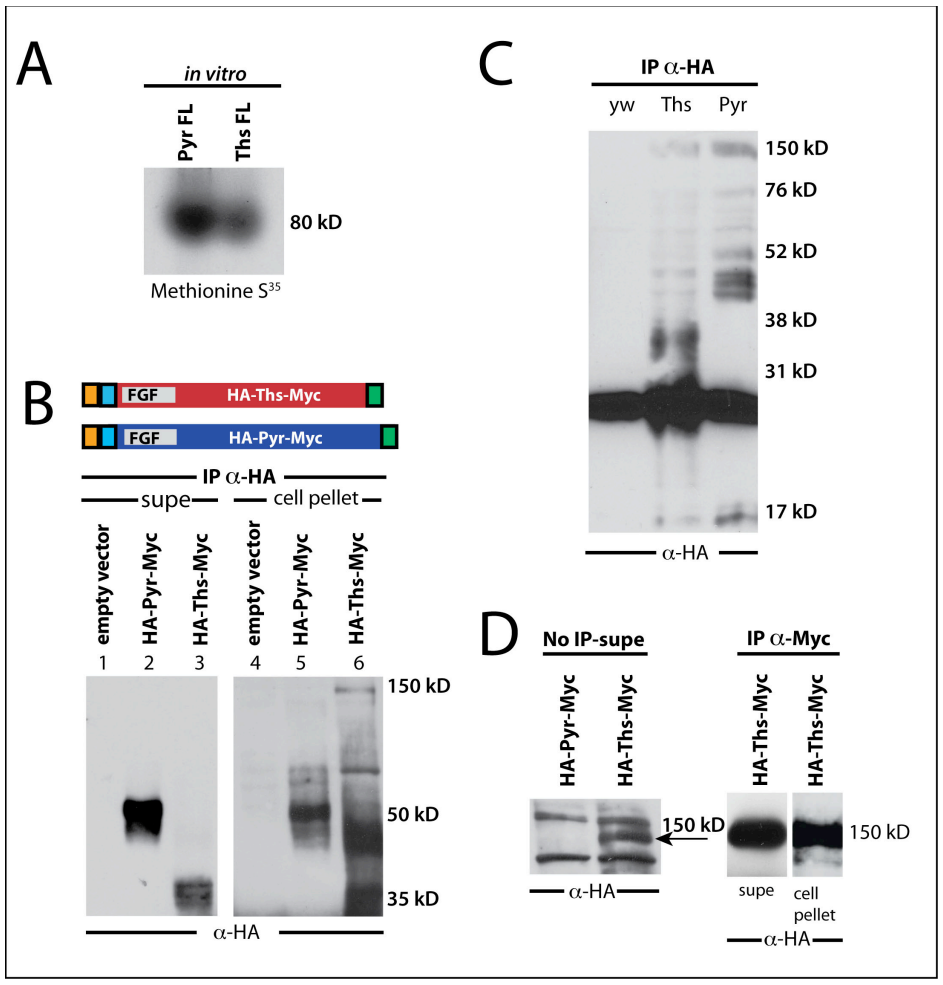

Figure 2 | FGFs are cleaved in S2 cell culture and embryonic extracts

(A) *In vitro* transcription/translation incorporating S^{35}Met into Pyr (left) and Ths (right) supports production of ~80 kD proteins, as predicted from the sequence. **(B)** Schematics of HA-Pyr-Myc and HA-Ths-Myc constructs showing the position of the signal sequence (orange box), N-terminal HA-tag (blue box), FGF domain, and C-terminal Myc tag (green box). Upon transfection of S2 cells, HA-Pyr-Myc and HA-Ths-Myc are secreted from cells as multiple bands around 50 kD for Pyr (lane 2) and 35 kD for Ths (lane 3), as detected by immunoprecipitation and immunoblot with anti-HA to track N-termini. Lane 1 and 4 are supernatant and cell pellet controls transfected with empty vector (i.e., pUASt).

Lane 5 and 6 are immunoprecipitations of HA-Pyr-Myc and HA-Ths-Myc from the cell pellet, showing cleaved forms are already detectable inside the cell. **(C)** Extracts from wildtype embryos (yw) or embryos overexpressing HA-Ths (Ths) or HA-Pyr (Pyr) with the 69B-GAL4 driver, immunoprecipitated with rat anti-HA and detected with mouse anti-HA reveal cleaved bands around 35kD for Ths and around 45kD for Pyr. **(D)** (Left Blot) Supernatant (i.e., cell culture medium) from HA-Pyr-Myc and HA-Ths-Myc, without immunoprecipitation, blotted with anti-HA antibody, shows a full-length band in the supernatant for Ths but not Pyr; the full-length Ths protein is present at much lower levels than the cleaved form and is only observable upon longer exposure; for instance, in **(B),** lane 3, it is not detected. (Right Blot) Immunoprecipitating with anti-Myc and blotting with anti-HA shows that the 150 kD band in Ths supernatant and cell pellet has both the N- and C-terminus connected.

Cleaved forms of Ths and Pyr are detected in embryonic extracts

To investigate whether Ths and Pyr proteins are also cleaved in the animal, we expressed tagged versions of these proteins (i.e., pUASt-HA-Ths and pUASt-HA-Pyr) in the embryo using the pan-ectodermal driver 69B-GAL4. Embryonic extracts were prepared from 1 gram of collected embryos, age 0-24 hour, and N-terminal protein species were isolated by using an anti-HA antibody for immunoprecipitation. Cleaved forms of both Ths and Pyr were detected in these samples, at ~35kD for Ths and ~45kD for Pyr (Fig. 2C). The ability to detect cleavage products from embryonic extracts of approximately the same size as those secreted in S2 cells suggests our analysis of Ths and Pyr processing in S2 cells may also be relevant to FGF function in the *Drosophila* embryo.

The C-terminal domain of Ths can be secreted, but not that of Pyr

Our ability to detect cleaved products in the S2 cell culture system, similar in size to those present in the animal, gave us confidence that we could use cell culture to obtain additional insights into these proteins. Therefore, we also examined the cell pellet fractions and found that cleaved N-terminal domains of Ths and Pyr are present inside the cell as well (Fig. 2B, lane 5 and 6). This result suggests that cleavage occurs inside the cell. To examine this possibility more closely, we assayed for the presence of full-length forms of Ths or Pyr inside and outside the cells.

In the HA-Ths-Myc cell pellet sample, in addition to the smaller potentially cleaved forms of Ths, we also detected a polypeptide of 150 kD, one that is much larger than the predicted size for Ths protein (~80kD) or that is observed when the cDNA is translated *in vitro* (Fig. 2B, lane 6, compare with Fig. 2A). To confirm that this protein species represented the full-length form of Ths, we immunoprecipitated with anti-Myc and blotted with anti-HA to identify both the C- and N-termini simultaneously. We observed that both N- and C-termini were connected in the 150 kD band in both the supernatant and cell pellet (Fig. 2D). Therefore, the 150 kD band probably represents full-length Ths, likely modified by glycosylation or other modifications that retard its mobility when assayed by SDS-PAGE and Western blot. Collectively, these results are consistent with the idea that the majority of Ths is cleaved intracellularly and secreted, while some full-length form is also secreted at lower levels.

Subceullular Localization of Pyr and Ths

We were not able to immunoprecipitate the predicted full-length Pyramus protein from the cell pellet or supernatant using a combination of anti-HA and anti-Myc antibodies, nor could we detect the cleaved C-terminus of Pyr by Western blot using an anti-Myc antibody; in contrast, full-length Ths could be detected in the supernatant at 150kD even in the absence of immunoprecipitation (Fig. 2D). Possible interpretations of these results are that (1) Pyr protein is processed from full-length to cleaved very quickly intracellularly leaving very little full length form available for detection, (2) that in S2 cells Pyr protein is never made as a "full-length" form, or alternatively, (3) the Myc epitope is not accessible. To address this question, we stained S2 cells expressing HA-Pyr-Myc constructs with either anti-HA or anti-Myc antibodies (Fig. 3). The stainings provided support that the N-terminus of Pyr (marked by the HA-tag) is secreted from the cell, as staining at the cell periphery was observed even in the absence of cell permeabilization (Fig. 3A, D). In contrast, the anti-Myc staining suggested that the C-terminus of Pyramus is present solely within cells, within an unidentified organelle, possibly an endosome (Fig. 3B). No anti-Myc staining could be observed for HA-Pyr-Myc in the absence of permeabilization (Fig. 3E). As a control for the accessibility of the Myc epitope, we used a C-terminally fused Pyr-GFP construct and anti-GFP antibody to confirm the location of the Pyr C-terminus. The anti-Myc and anti-GFP stainings of HA-Pyr-Myc and Pyr-GFP, respectively, exhibit the same intracellular staining that is lost in the absence of permeabilization (Fig. 3B,C,E,F). These data suggests that the C-terminus of Pyramus is translated, but that the full-length and C-terminus of the protein stays within the cell and is not secreted. Stainings for Ths confirmed what was seen in Western blots. The N-terminus was present both

inside the cell (Fig. 3G) and at the membrane in non-permeabilized cells (Fig. 3J). Stainings marking the C-terminus of Thisbe with anti-Myc and anti-GFP support the idea that the C-terminus Thisbe protein is secreted, although this could represent either a full-length or cleaved form (Fig. 3H,I,K,L). Therefore, we propose that there may be a difference in the number of forms secreted for Ths versus Pyr: Ths may be secreted as both a full-length and a cleaved form, whereas Pyr is only secreted as a cleaved form with the C-terminus being retained intracellularly.

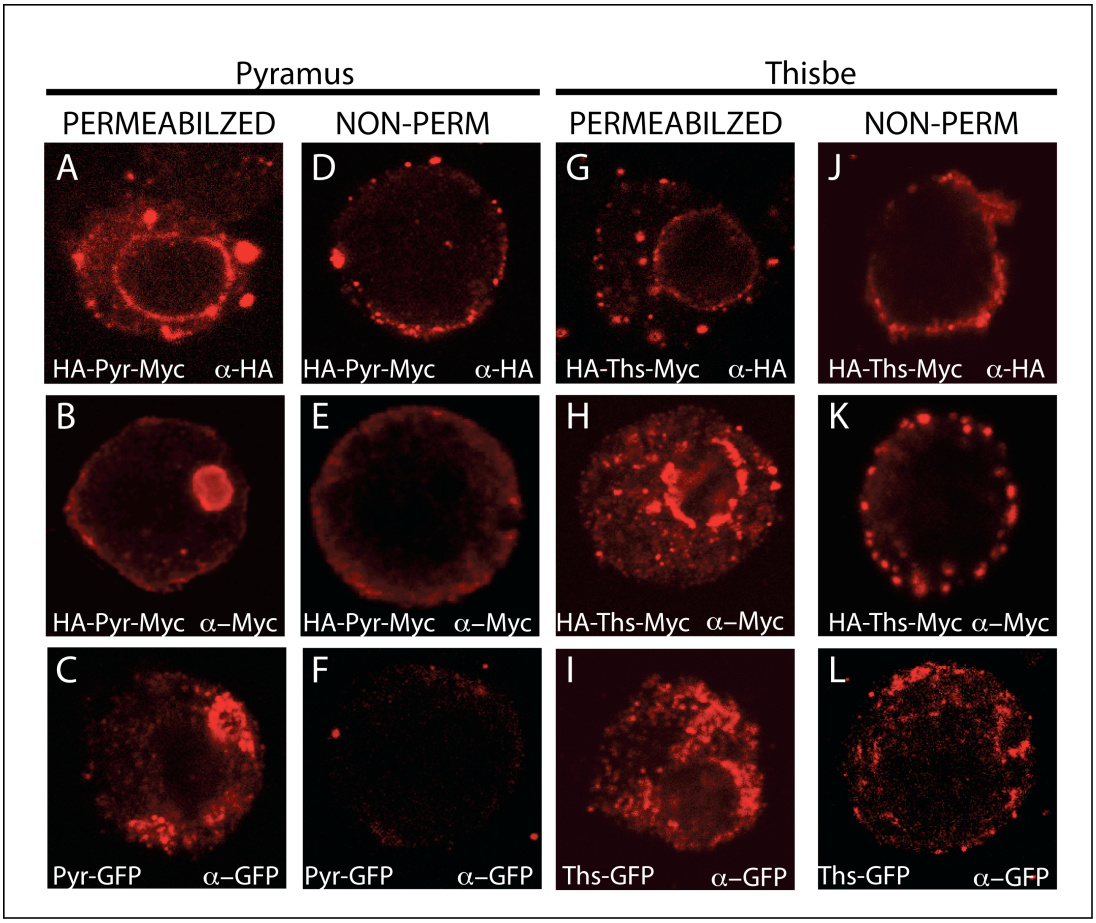

Figure 3 | Visualization of N- and C-termini in S2 cells shows a difference in the subcellular localization of Ths and Pyr C-terminus.

S2 cells were transfected with the indicated pUASt.HA-Pyr-Myc, pUASt.Pyr-GFP, pUASt-Ths-GFP or pUASt.HA-Ths-Myc constructs (see methods), and immunofluorescence was conducted using anti-HA, anti-Myc, or anti-GFP antibodies (all "red"). HA-Pyr-Myc was stained with anti-HA to see the N-terminus **(A,D)** where predominant ER staining was seen inside the cell **(A)** while only membrane staining was seen under non-permeabilizing conditions **(D)**. The C-terminus of Pyr was visualized with anti-Myc for HA-Pyr-Myc **(B,E)** or anti-GFP for Pyr-GFP **(C,F)**. The C-terminus of Pyr inside the cell was localized to small, non-nuclear vesicles, which may be endosomal in character **(B,C)**. No Pyr C-terminus was visualized outside of the cell **(E,F)**. Anti-HA was used to visualize the HA-Ths-Myc N-terminus under permeabilizing **(G)** and non-permeabilizing conditions **(J)**, revealing ER staining around the nucleus inside the cell **(G)** and proteins attached to the cell membrane **(J)**. Anti-Myc **(H,K)** and anti-GFP **(I,L)** were both used to visualize the Ths C-terminus of either HA-Ths-Myc or Ths-GFP. Again, ER staining was seen inside the cell **(H,I)** and membrane staining was observed under non-permeabilized conditions **(K,L)**.

Truncation constructs reveal the FGF domain alone is sufficient for function

In order to reconcile the biochemical evidence for cleaved forms with the endogenous function in the embryo, we made a series of N-terminally, HA-tagged truncation constructs and used the site-directed transgenic method to insert all transgenes in the same genomic location on the third chromosome, 86FB, to minimize positional effects (BISCHOF *et al.* 2007). We cannot be sure that the act of truncation itself does not impart differences in the stabilities of the produced proteins; in fact, stability of the proteins (possibly regulated by

cleavage events) may be one mechanism by which their activities are regulated. However, by minimizing positional effects on the transgene, we standardized expression levels for each of the constructs to the best of our abilities.

During stage 10 to 11, FGF signaling through Htl contributes to differentiation of the mesoderm into specific cell types, including the pericardial cells of the future heart tube and dorsal somatic muscle (BEIMAN *et al.* 1996). At this stage, *pyr,* and to a lesser extent *ths* as well, is expressed in the ectoderm overlying the developing heart cells (STATHOPOULOS *et al.* 2004). Signaling through Htl, presumably by wild type endogenous levels of Pyr/Ths, supports the generation of three Eve-positive cells per hemisegment (Fig. 4A and (MICHELSON *et al.* 1998b)), while overexpression of either Ths or Pyr leads to the expansion of this cluster up to 20 cells (KADAM *et al.* 2009).

We used the expansion of the Eve-positive cell cluster as a functional readout to test the function of Ths or Pyr tagged, truncated proteins when overexpressed in the ectoderm with 69B-GAL4. First, addition of HA and Myc tags to Ths and Pyr did not affect the ability of Ths and Pyr to cause an expansion of the Eve-positive cluster (Fig. 4B, F). Furthermore, of three truncation constructs engineered for Ths, two were functional (HA-Ths$^{1-158}$ and HA-Ths$^{1-403}$) and one was not (HA-Ths$^{1-261}$). The two that were functional were also secreted, as confirmed by expression in S2 cells, while the construct that was not functional was not secreted (Fig. 4C, D, E); the non-functional truncation may disrupt an essential secondary structure required for proper folding and in turn secretion. Remarkably, the small HA-Ths$^{1-158}$ was secreted and functional, yet this polypeptide contains little more than the FGF

domain alone. Together, these data suggest that the FGF domain alone is sufficient for function of Ths and that secretion is also required for function.

Three truncated constructs were engineered for Pyr as well: Pyr^{1-220}, Pyr^{1-348}, and Pyr^{1-466}. Similar to the results from Ths, the two Pyr truncations that were secreted were also functional (Pyr^{1-348} and Pyr^{1-466}) (Fig. 4H, I), while Pyr^{1-220} was neither secreted nor functional (Fig. 4G). Unlike Ths, the fact that Pyr^{1-220} was not functional suggests that the shortest functional Pyr construct requires additional sequence besides the FGF domain. It may be possible to make a shorter functional construct of Pyr; the Pyr^{1-220} construct may have been terminated in a location critical for proper folding. Nevertheless, the functionality of Pyr^{1-348} suggests aa residues 349-766 are not required for activity.

Differential range-of-action resulting from limiting the source of FGF

Previous studies on the function of Ths and Pyr have speculated that a possible difference in their signaling capacity is due to either a differential range-of-action of the ligands diffusing from their source of expression or due to an unequal potency of activating the receptor (e.g., receptor-binding affinity) (KADAM *et al.* 2009). In order to address these unanswered questions and to gain more sensitivity than was possible with the pan-ectodermal 69B-GAL4 Eve-positive cluster assay (Fig. 4), we used a different driver, ZenKr-GAL4, which drives expression in a subset of the embryonic dorsal ectoderm

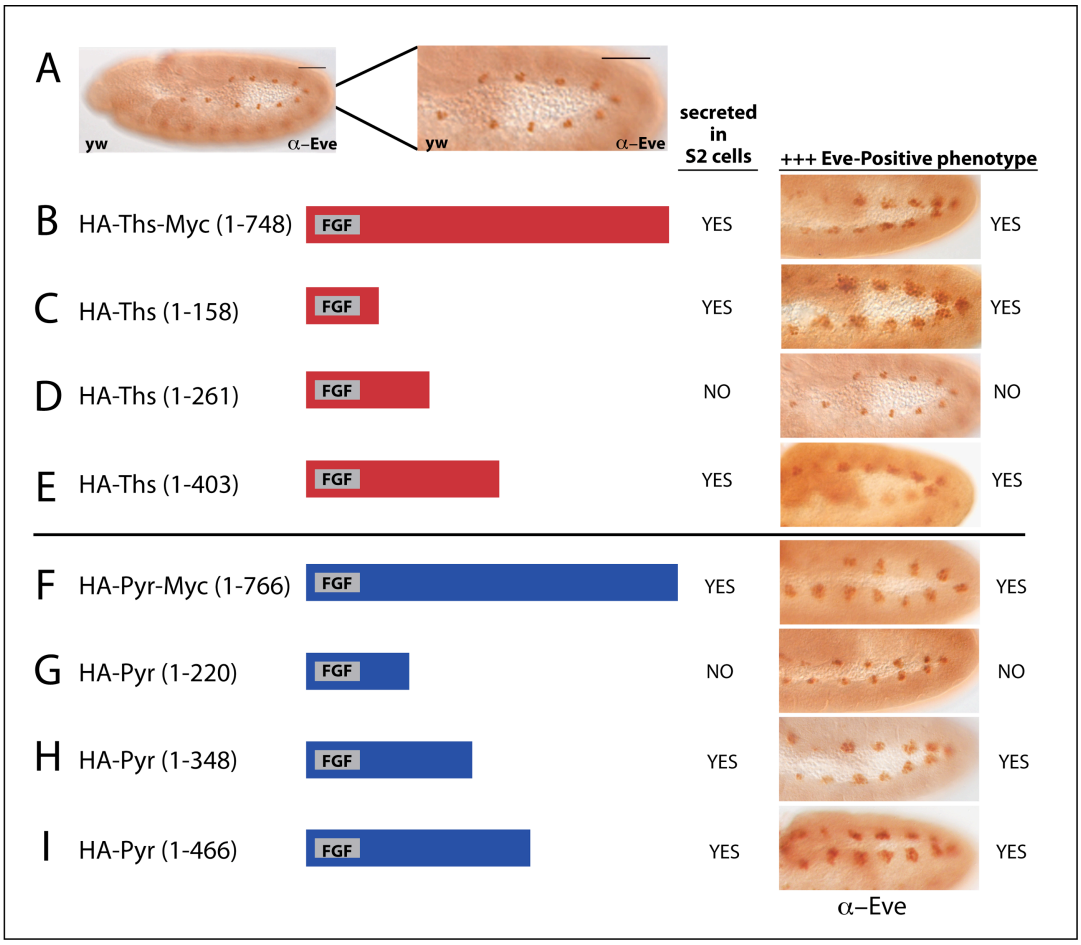

Figure 4 | Ths and Pyr truncation constructs that support the production of secreted proteins in cell culture are also functional in the embryo

(A) Stage 11, wild type embryos, lateral view, stained with anti-Eve antibody show Eve-positive staining in three cells per hemisegment. The enlargement of the Eve-positive area is 1.8x. **(B-I)** pUASt-HA-Ths and pUASt-HA-Pyr full-length and truncated construct schematics; assay for secretion was conducted in S2 cell culture. Embryos overexpressing indicated constructs with 69B-GAL4 were stained with anti-Eve antibody to score for FGF activity. **(B)** Overexpression of full-length Ths results in more Eve-positive cells (Eve+++). **(C, E)** HA-Ths$^{1-158}$ and HA-Ths$^{1-403}$ are both secreted and Eve +++, but **(D)** HA-Ths$^{1-261}$ is

not secreted and does not support more Eve-positive cells. **(F)** Overexpression of full length Pyr also results in Eve+++. **(G)** HA-Pyr$^{1-220}$ is not secreted and does not give more Eve-positive cells, but **(H, I)** HA-Pyr$^{1-348}$ and HA-Pyr$^{1-466}$ are both secreted and exhibit the Eve+++ phenotype.

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starting just before stage 9; at this stage embryos have undergone 50% of germ band elongation and expression supported by the driver is localized to the posterior (Fig. 4A and zen.VRE.Kr-GAL4 FRASCH 1995)). For each construct, the number of Eve-positive cells per cluster in each hemisegment of 25 embryos was counted, averaged and compared. The clusters were tracked within embryo hemisegments as indicated by the numbers on Fig. 5A. ZenKr-Gal4 supports expression in clusters 4-7 (as seen in inset Fig. 5G') and this domain is represented on the graphs by a shaded gray box in the background.

ZenKr-GAL4 driving full length HA-Ths-Myc and HA-Pyr-Myc both resulted in extra Eve-positive cells outside the expression domain supported by the ZenKr enhancer (i.e., clusters 1-3 and 8-11) (Fig. 5A, D). Furthermore, expression of HA-Pyr-Myc resulted in more Eve-positive cells within every hemisegment as compared with expression of HA-Ths-Myc (Fig. 5G). One interpretation of this result is that Pyr may be more potent in its activation of the Htl receptor and another is that the Pyr protein is secreted at higher levels or is more stable than Ths. Both HA-Pyr-Myc and HA-Ths-Myc supported more Eve-positive cells at the source (i.e., clusters 4-7; gray box) and tapered off in a graded manner to more distant clusters (Fig. 5G).

Expressing HA-Ths$^{1-158}$ in the ZenKr domain resulted in a surprising result: when compared to full length HA-Ths-Myc, HA-Ths$^{1-158}$ supported the expression of many more Eve-positive cells in each hemisegment, even those farthest from the source (Fig. 5B, H, especially clusters 1 and 11). Compared to full-length HA-Ths-Myc, HA-Ths$^{1-158}$ also had a dramatically different profile of Eve-positive cell numbers; instead of peaking at the source and dropping off in a graded manner, there was close to maximum expression of Eve supported in almost every hemisegment (Fig. 5H). In contrast, the other truncated Ths construct, HA-Ths$^{1-403}$ showed an increase of Eve positive cells as compared to HA-Ths-Myc within clusters at the source yet tapered off in activity in more distant clusters, a profile similar to that of the full length construct (Fig. 5 C, H). In summary, two changes in trend were associated with constructs HA-Ths$^{1-158}$ and HA-Ths$^{1-403}$ compared with full-length Ths: (I) flattened profile versus (II) increase peak yet graded profile, respectively.

With the overexpression of the ligands limited to the domain of ZenKr-Gal4 expression, we favor the idea that supernumerary Eve-positive cells in hemisegments outside this domain would most likely result from an increase in diffusion of the ligands from their source of ectopic expression or decreased receptor-mediated endocytosis; however we cannot dismiss an alternate scenario in which this result is supported by the Ths$^{1-158}$ protein being more stable than other constructs. In either case, our results suggest the C-terminus of Ths has an inhibitory function (for example, either affecting stability, endocytosis or diffusion) and possibly that cleavage of Ths plays a regulatory role in increasing the ability of this protein to support activation of FGFR.

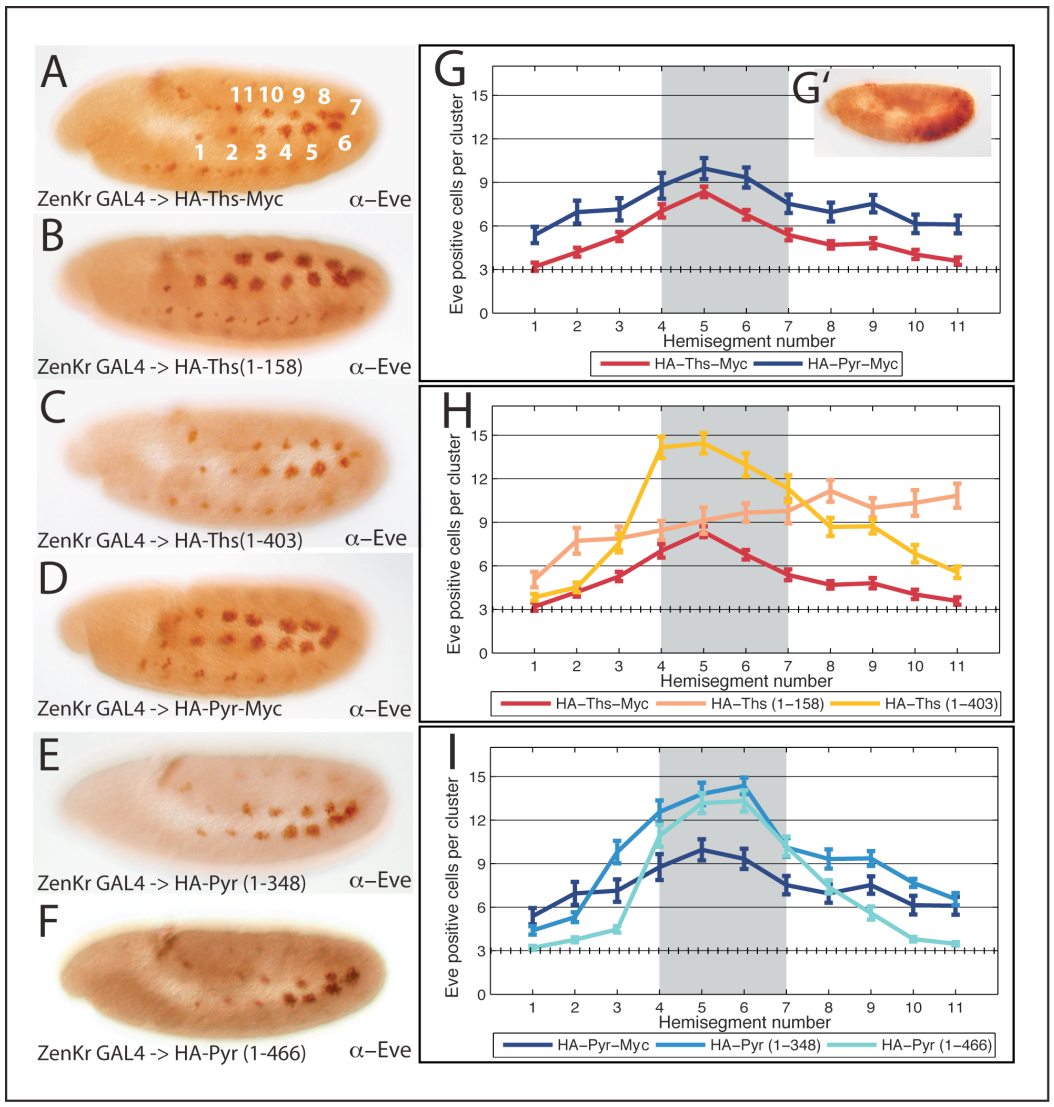

Figure 5 | Restricting the source of full-length and truncated Ths and Pyr constructs reveals a functional difference

(A-F) Immunohistochemistry on stage 11 embryos, lateral view, all constructs driven with ZenKr-GAL4; embryos were stained using an anti-Eve antibody. **(A)** White numbers indicate position of numbered Eve-positive clusters; ZenKr-GAL4 supports expression in clusters 4-7. **(A-F)** Eve staining reveals additional Eve-positive cells outside the ZenKr

domain for **(A)** HA-Ths-Myc **(B)** HA-Ths$^{1-158}$ **(C)** HA-Ths$^{1-403}$ **(D)** HA-Pyr-Myc **(E)** HA-Pyr$^{1-348}$ **(F)** HA-Pyr$^{1-466}$. **(G,H,I)** Eve-positive cells per cluster were counted in each hemisegment for 25 embryos per construct tested and averaged. Error bars indicate standard error. **(G)** The hatched line at "3" represents the wild-type level of Eve-positive cells. The gray box represents the source of expression supported by ZenKr-GAL4. Plot of Eve-positive cells generated by ZenKr-GAL4 → pUASt-HA-Ths-Myc as compared to ZenKr-GAL4 → pUASt-HA-Pyr-Myc shows that Pyr has greater functional activity than Ths. Ths and Pyr both give a graded output of Eve-positive cells with the most cells in the source domain. **(G')** ZenKr-GAL4 driving UAS-lacZ and stained with anti-βgal shows the domain of the driver in the posterior dorsal ectoderm of the embryo. **(H)** ZenKr-GAL4 → pUASt-HA-Ths$^{1-158}$ does not have the same Eve-positive profile, instead it results in more Eve-positive cells in clusters 8-11. ZenKr-GAL4 → pUASt-HA-Ths$^{1-403}$ has increased activity locally but similar levels of function to HA-Ths-Myc at long-range **(I)** ZenKr-GAL4 → pUASt-HA-Pyr$^{1-348}$ and ZenKr-GAL4 → pUASt-HA-Pyr$^{1-466}$ both retain a graded profile of Eve-positive cells, although HA-Pyr$^{1-348}$ supports more Eve-positive cells in distant clusters 8-11 as compared to HA.-Pyr$^{1-466}$.

We also expressed both functional truncations of Pyr with ZenKr-GAL4 and found that overexpression of both HA-Pyr$^{1-348}$ and HA-Pyr$^{1-466}$ supported additional Eve-positive cells (Fig. 5E, F). For both HA-Pyr$^{1-348}$ and HA-Pyr$^{1-466}$ the profile was similar to trend II seen for HA-Ths$^{1-403}$: increased peak levels but graded profile (Fig. 5I). Our hypothesis is that only the N-terminal Pyr cleavage product is secreted and the C-terminus remains

intracellular so truncated Pyr would be expected to support a similar response to expression of full-length Pyr due to essentially the same net protein fragment being secreted. We do indeed see a similar profile of expression (i.e., graded profiles). However, the outputs observed for the two Pyr truncation constructs and full-length Pyr are not identical; we suggest these differences may be due to differences in protein production (e.g., stability, differential processing inside the cell, and/or rate of secretion).

Pyr truncation constructs are modified

In S2 cell supernatants, truncated Pyr constructs, HA-Pyr$^{1-348}$ and HA-Pyr$^{1-466}$ run at the same size on Western blots despite the larger construct containing 118 more amino acids (Fig. 6A). To understand if this was due to post-translational modifications we expressed both truncated constructs in a cell-free transcription/translation system and compared them to the size of the bands immunoprecipitated with anti-HA from the S2 cell supernatant. In S2 cells, HA-Ths$^{1-158}$ and HA-Pyr$^{1-466}$ ran similar to their predicted sizes (20kD and 50kD, respectively) but HA-Pyr$^{1-348}$ ran larger than its predicted size (at 50kD instead of 35kD), indicating that it may be glycosylated or otherwise modified (Fig. 6A). These modifications could correspond to the predicted O-glycosylation sites between aa 177 and 201 in Pyr (Fig. 1). When HA-Pyr$^{1-348}$ was expressed in the cell-free system (without the opportunity for post-translational modifications including glycosylation), it ran at its predicted size of 35 kD (Fig. 6B). HA-Pyr$^{1-466}$ likely contains the same modifications (and would likely also run larger than predicted, possibly around 65kD), but we hypothesize it is subsequently cleaved to the smaller 49/50 kD size of secreted Pyr (Fig. 6C, predicted sequential steps 1-3). Carbohydrate modifications such as glycosylation can significantly

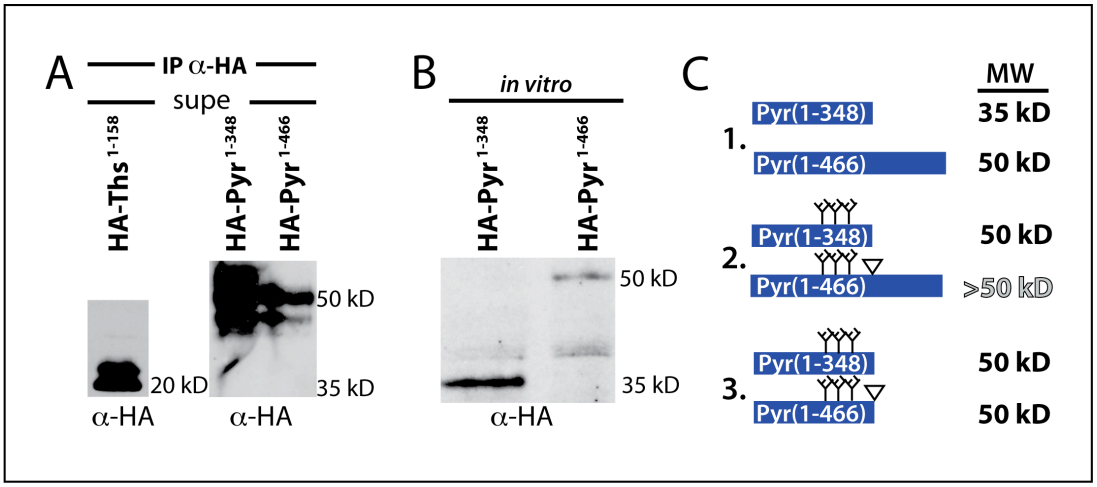

Figure 6 | Post-translational modifications contribute to the observed molecular weight of Pyramus

(A) Supernatants from S2 cultured cells, immunoprecipitated with anti-HA, blotted with anti-HA. HA-Ths$^{1-158}$ runs at the expected size of 20kD, but HA-Pyr$^{1-348}$ runs larger than predicted, at the same size as HA-Pyr$^{1-466}$. **(B)** *In vitro* transcription/ translation of HA-Pyr$^{1-348}$ (left lane) and HA-Pyr$^{1-466}$ (right lane) blotted with anti-HA, show HA-Pyr$^{1-348}$ is likely post-translationally modified *in vivo*. **(C)** Schematic showing predicted events to explain the results in A and B: 1.) HA-Pyr$^{1-348}$ and HA-Pyr$^{1-466}$ are both translated to their predicted sizes as seen *in vitro* in **(B)**. 2.) HA-Pyr$^{1-348}$ and HA-Pyr$^{1-466}$ are both modified post-translationally, likely by the addition of carbohydrate chains which increase their molecular weight to 50kD and >50kD (unobserved). 3.) HA-Pyr$^{1-466}$ is probably subsequently cleaved to decrease its molecular weight to 50kD. Small branching trees represent modifications and the inverse triangle represents a predicted cleavage site.

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affect the secretion, diffusion and binding capabilities of ligand proteins and the difference in modifications between Ths versus Pyr could contribute to their individual capabilities.

Ths-Pyr chimeras reveal differences between Ths and Pyr C-termini

Results from the truncation constructs showed that eliminating the C-terminus from Ths has a different effect than eliminating the C-terminus from Pyr (see Figure 5H,I). To further address the differences in the N- and C-termini of Ths and Pyr, we made chimeric proteins containing the N-terminus from one ligand and the C-terminus from the other, ThsN-PyrC and PyrN-ThsC. Both chimeras were secreted and functional, giving extra Eve-positive cells when driven by 69B-GAL4 (Fig. 7A). In S2 cell culture, both chimeras were processed and secreted as cleaved forms. When the N-terminus of each chimera is detected using an anti-HA antibody, we find that ThsN-PyrC is cleaved into a 50/52 kD doublet, and one band at 30 kD (Fig. 7B lane 4); as a result, it may contain cleavage sites and/or modifications derived from both Pyr and Ths. PyrN-ThsC is present as a small doublet around 30/35 kD, indicating it is likely cleaved and modified according to information derived from its Ths sequence (Fig. 7B lane 3 compared with lane 2). Importantly, this result shows that the cleavage is dependent upon the specific Ths or Pyr ligand sequence used and swapping sequence outside the FGF-domain allows us to see how the FGF-domain of one ligand acts in the context of processing like the other ligand.

The chimeras were also driven by ZenKr-GAL4 and the Eve-positive cells counted as was previously done for the truncated constructs. ThsN-PyrC supported more Eve-positive cells than HA-Ths and furthermore had the same altered profile as HA-Ths$^{1-158}$ (Fig. 7 C, E). PyrN-ThsC had decreased Eve-positive cell clusters compared to the HA-Pyr$^{1-348}$, and was similar to full-length Pyr (Fig. 7 D, F). These results demonstrate that the Pyr C-

terminus does not have the same inhibitory effect on the Ths N-terminus that the Ths C-terminus does.

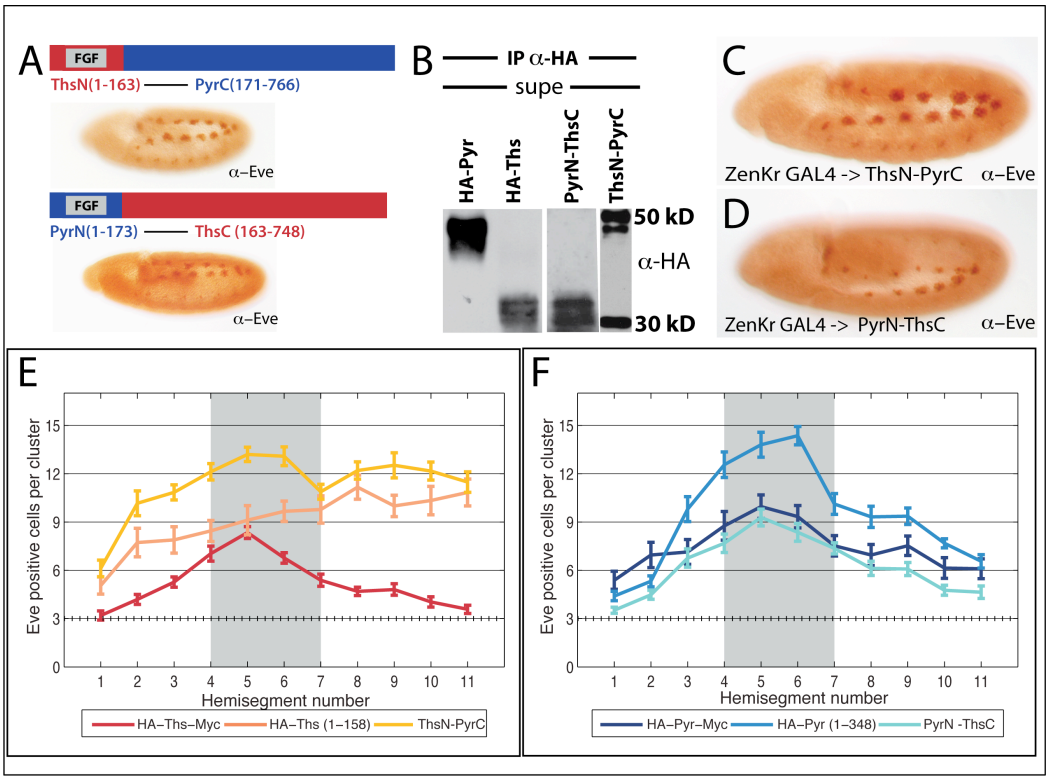

Figure 7 | Ths-Pyr Chimeras highlight inhibitory activity of Ths C-terminus

(A) Schematics of ThsN-PyrC and PyrN-ThsC chimeric constructs and stage 11 embryos with 69B-GAL4 driving expression and function monitored with anti-Eve. Both chimeras give the +++Eve phenotype, meaning they support FGF activity. **(B)** Both chimeras are secreted, but cleaved differently in S2 cells. Supernatant immunoprecipitated with anti-HA and blotted with anti-HA, shows PyrN-ThsC is cleaved in a manner similar to Ths, while ThsN-PyrC may have both Pyr and Ths-derived cleavage sites. **(C,D)** Anti-Eve in stage 11 embryos with each chimera driven by ZenKr-GAL4 shows +++Eve cells in clusters outside the ZenKr domain for **(C)** pUASt-ThsN-PyrC and **(D)** pUASt-PyrN-ThsC. **(E,F)**

Comparisons of Eve-positive cells per cluster in each hemisegment, scored and averaged as in Fig. 5. **(E)** ZenKr-GAL4 → ThsN-PyrC gives more Eve-positive cells than HA-Ths-Myc, suggesting that the Pyr C-terminus does not inhibit the Ths N-terminus the way that the Ths C-terminus does. **(F)** ZenKr-GAL4 → PyrN-ThsC has fewer Eve-cells than ZenKr-GAL4 → HA-Pyr$^{1\text{-}348}$, and is similar to full-length HA-Pyr-Myc indicating the Ths C-terminus can likewise inhibit the Pyr N-terminus.

Deleting putative cleavage region renders Ths non-cleavable

In order to address whether the Ths cleavage is necessary for proper function, we attempted to engineer a Ths construct with a deletion of the putative cleavage sites to create an un-cleavable form of the ligand. To determine which region to delete, we considered the size of the 30-35 kD bands secreted in S2 cells and dibasic/multibasic Arginine/Lysine sequences characteristic of the recognition sites of Furin-like proteases responsible for processing proproteins of other growth factors like Dpp into mature forms (RHOLAM and FAHY 2009). Sequences underlined with a red line (Fig. 8A) contain basic amino acids stretches of [R/K]-[X_n]-[R/K] where X indicates any amino acid residue and *n* is 0, 2, 4, or 6, which is the consensus recognition sequence for Furin-related proprotein convertases (RHOLAM and FAHY 2009).

We deleted a 72 aa region containing 5 putative cleavage sites to generate the construct HA-Ths $^{\Delta 261\text{-}333}$ –Myc (Fig. 8A). When HA-Ths $^{\Delta 261\text{-}333}$-Myc was immunoprecipitated from S2 cell culture with anti-HA antibody, the full-length band became much more prominent than that associated with HA-Ths-Myc (Fig. 8B, lane 3 vs. 2), although cleavage products

still remain. We then extended the deleted region 23 aa further to remove one additional weak match to the Furin consensus sequence to make HA-Ths$^{\Delta 261-356}$-Myc. The cleaved bands were further diminished, although one band of ~35kD was still detectable (Fig. 8A and 8B, lane 4). The simplest interpretation is that these 95 amino acids include most of the relevant cleavage sites for Ths, and deleting them results in a shift of the dominant Ths protein species to the full-length form.

The function of HA-Ths$^{\Delta 261-356}$-Myc was tested using both the 69B-GAL4 assay and the ZenKr-GAL4 assay. We hypothesized that the drastic reduction of cleaved Ths and the presence of increased full-length Ths would result in either dramatically less function if cleavage were activating or dramatically more function if cleavage were inactivating. Surprisingly, the result was neither of these extremes, but instead HA-Ths$^{\Delta 261-356}$-Myc was still able to generate supernumerary Eve positive cell clusters like the other constructs (Fig. 8C) and when the source of expression was limited using ZenKr-GAL4, the graded output of Eve positive cells was flattened as compared to HA-Ths-Myc (Fig. 8D, E), similar to the profile of HA-Ths$^{1-158}$, yet not as potent an activator (compare with Fig. 5H).

Thus by deleting 95 aa, we can affect the proteolytic processing of the Ths protein such that the majority of the protein is present as full-length. Because this construct supports detectable activity, we suggest it is unlikely that cleavage is required for this activity. However, we cannot dismiss this possibility as some cleaved product is detected and the effective dose of FGFs is often very small; the remaining cleaved products in HA-Ths$^{\Delta 261-356}$-Myc may be sufficient to function when overexpressed at high levels with GAL4

drivers. Nevertheless, HA-Ths$^{\Delta 261\text{-}356}$-Myc supports an expanded profile similar to HA-Ths$^{1\text{-}158}$; perhaps the flattened output is an indication that both of these proteins are not endocytosed properly (see Discussion). It is possible that cleavage is required before ligands can be effectively endocytosed from the extracellular space.

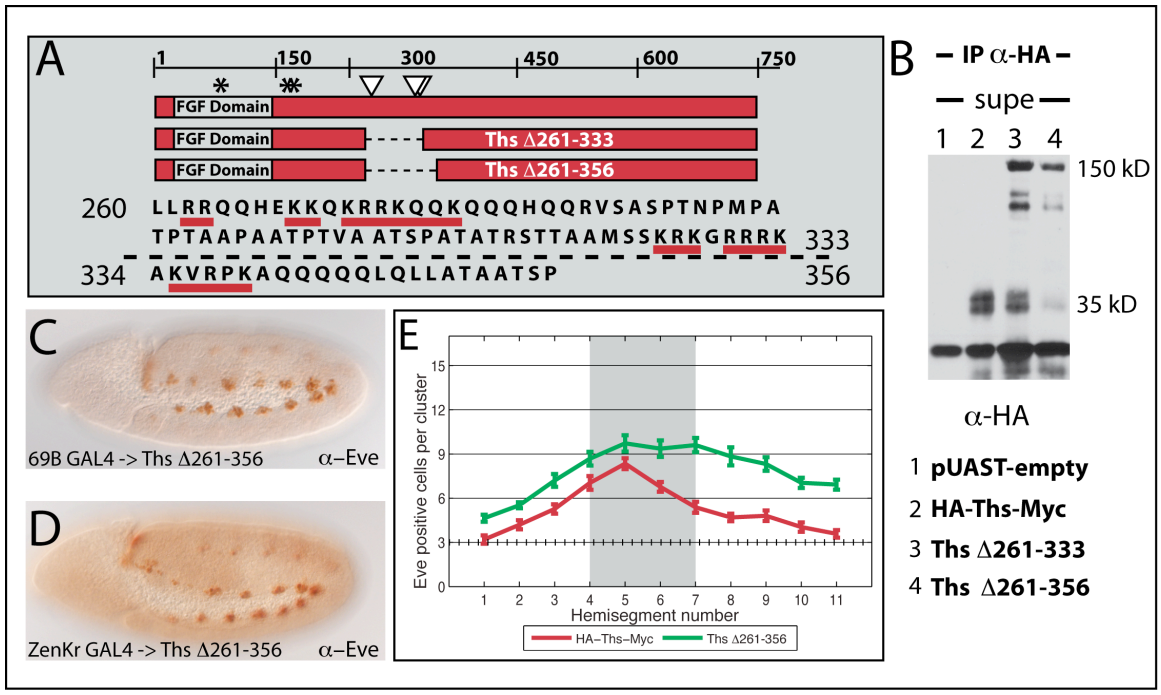

Figure 8 | Cleavage of Ths can be prevented through deletion of internal amino acids

(A) Thisbe amino acid sequence deleted in Ths$^{\Delta 261\text{-}333}$Myc, including 5 potential cleavage sites, underlined in red; below the dotted black line are the amino acids that differ between the two deletion constructs, Ths$^{\Delta 261\text{-}333}$Myc and Ths$^{\Delta 261\text{-}356}$Myc, including 1 additional potential cleavage site, underlined in red. **(B)** Western blot of anti-HA immunoprecipitations from supernatant of cells transfected with pUASt-empty, HA-Ths-Myc, HA-Ths$^{\Delta 261\text{-}333}$Myc, and HA-Ths$^{\Delta 261\text{-}356}$Myc. HA-Ths-Myc was loaded 5x less than the other samples to equalize the exposure while resolving the double-band at 35kD.

Ths$^{\Delta 261\text{-}333}$Myc (lane 3) is still partially cleaved but has increased full-length protein and (lane 4) Ths$^{\Delta 261\text{-}356}$Myc has less cleavage and more full-length product, as compared to HA-Ths-Myc (lane 2). **(C)** 69B-GAL4 driving Ths$^{\Delta 261\text{-}356}$Myc results in more Eve-positive cells in every hemisegment, as compared to wildtype. **(D)** ZenKr GAL4 driving Ths$^{\Delta 261\text{-}356}$Myc results in extra Eve-positive cells outside the source of expression. **(E)** Eve-positive cells counted in 11 hemisegments for 25 embryos and averaged as in Figure 5. The gray box represents the source of expression supported by ZenKr-GAL4. As compared to HA-Ths-Myc, Ths$^{\Delta 261\text{-}356}$Myc has a decreased gradient of functional output, resulting in a flatter profile.

Discussion

Proteolytic processing regulates many signaling molecules in both vertebrates and invertebrates, and unlocking the mechanisms behind regulatory cleavage events has been an on-going effort for each protein. In 1990, Dpp was first reported to be cleaved in S2 cells (PANGANIBAN *et al.* 1990), and recent studies have continued to piece together the details of the maturation of the Dpp protein (KÜNNAPUU *et al.* 2009). Our current analysis is the first evidence for cleavage of Ths and Pyr FGF ligands for the Htl FGFR. In this study we have shown that Ths and Pyr proteins are both cleaved and secreted from S2 cells and cleaved Ths and Pyr can be detected in embryonic extracts. Truncated ligands with the N-terminal FGF-domain are functional. Additionally, spatially restricting the source of FGF ligands and using domain-swapped chimeras revealed that the C-terminus of Ths has an inhibitory capability while the C-terminus of Pyr does not.

The roles of Ths and Pyr C-terminal domains are different

The C-terminus of Ths, but not that of Pyr, can be detected extracellularly in tissue culture cells, suggesting that the processing situation for Ths is likely different to that for Pyr and raising the possibility that the function of the C-termini of these proteins has diverged. Even though we can detect cleaved forms of Ths inside the cell, indicating that cleavage likely occurs intracellularly, we can also detect both full-length and cleaved forms of Ths outside of the cell. Why would both forms be secreted if only the cleaved form was necessary to participate in receptor binding? In the case of Notch, two of the processed forms remain non-covalently associated and must be further processed for release (BLAND *et al.* 2003). In the case of Ths, the presence of both the unprocessed form and the processed form outside the cell creates the opportunity for an interaction.

While expression of full-length Ths has a graded range-of-action, we were surprised to find that truncated Ths has an extended range-of-action which suggests that the C-terminal domain is inhibitory. The mechanism by which the C-terminus of Ths accomplishes its inhibitory role remains an intriguing question. Does the C-terminus affect the rate of cleavage, the diffusion range, the rate of endocytosis, or might it physically interact with the N-terminus to directly inhibit FGF binding to the receptor? The Ths C-terminus has a similar effect on the Pyr FGF domain-containing N-terminus, as the PyrN-ThsC chimera is functionally restricted compared to the shorter Pyr^{1-348}. In contrast, the Pyr C-terminus does not have the same effect on the Ths N-terminus, as ThsN-PyrC is highly potent and diffuses in an unrestricted manner, similar to truncated Ths^{1-158}.

Our data supports the view that Pyr is processed inside the cell and that only the N-terminal cleaved form is released; therefore the Pyr C-terminus may only have a cell autonomous effect and likely does not affect the secreted protein directly once it is released. The local potency of HA-Pyr-Myc is less than the truncated Pyr constructs; so we propose cleavage of Pyr inside the cell may be a rate-limiting step.

Our results are also consistent with the idea that Pyramus could contain a transmembrane domain, as predicted, although our inability to follow the Pyr C-terminus has prevented us from confirming this prediction. Although we have not yet uncovered a specific role for the Pyr C-terminus, of note is the fact that the C-terminal domain of Pyr exhibits homology to the intracellular human protein Trinucleotide Repeat-Containing 15 (Tnrc15) implicated in Parkinson's disease (18% identity vs. 38% similarity over an approximately 500 aa stretch; data not shown) (LAUTIER *et al.* 2008). Tnrc15 interacts with Grb10, which, in turn, interacts with EGFR, MAP2K1 and many other signaling molecules (GIOVANNONE *et al.* 2003). This homology suggests the Pyr C-terminus may function inside the cell to regulate signaling, a function that likely is distinct from that of the Ths C-terminal domain.

Processing of FGF ligands: proteolytic cleavage and post-translational modification

Further studies will be required to understand the role that processing of Ths and Pyr plays in the regulation of FGF signaling in *Drosophila*. The proteases responsible for the processing may themselves be spatially or temporally regulated at the transcriptional level,

or separated into different subcellular compartments. Additional regulation of FGF signaling activity by proteases, which alter ligand activity and/or diffusion, could explain how FGFs are able to perform so many distinct functions in animals. For example, the diffusion range of Ths and Pyr, possibly regulated by proteolytic cleavage, could be important to support different functions. For instance, recently we have learned that during gastrulation Ths and Pyr guide the symmetric collapse of the mesoderm first and subsequently control intercalation of cells required for monolayer formation (AMY MCMAHON 2010). For collapse, a long-range signal might be required, whereas to support the small cell movements of intercalation a short-range signal may be more effective. In addition, findings from the TGF-β signaling family show in some cases ligands are differentially processed in a tissue-specific manner. Differential processing of BMP-4 by Furin proprotein convertases results in multiple ligand forms that exhibit differences in stability and ability to act at a distance in *Xenopus* assays (CUI *et al.* 2001; DEGNIN *et al.* 2004). Recent results on the BMP-2/4 homolog, Dpp, have found not only is Dpp processed in a tissue-dependent manner but different cleavage products are also required to provide sufficient function for wing and leg versus gut development (KÜNNAPUU *et al.* 2009; WHARTON and DERYNCK 2009). These examples highlight the importance of ligand processing as a key mechanism used by the cell to control ligand presentation and tissue-specific signaling.

Range of action: diffusion and regulated endocytosis

Diffusion range is very important for most secreted signaling molecules, and this range may be influenced by post-translational modifications, proteolysis, or interactions with

other secreted molecules. Recent work from zebrafish has shown that the common homolog of Ths and Pyr, FGF8, can act as a morphogen and spread from its source in the mid-hindbrain boundary by simple diffusion (YU *et al.* 2009b). A slower-moving species of FGF8 was also detected, which is thought to be interacting with heparin sulfate proteoglycans (HSPGs) in the extracellular matrix. HSPGs are extracellular matrix and cell surface macromolecules that consist of a protein core to which heparin sulfate (HS) glycosaminoglycan (GAG) chains are attached. HSPGs are required as a co-receptor in vertebrate FGF signaling and might also be involved in *Drosophila* FGF signaling (LIN *et al.* 1999). Alternatively, the excessive glycosylation implicated in the molecular weight of full-length Ths (150kD compared to the predicted size of 82 kD) implies that the fully modified Ths molecule may likely be slow diffusing even without binding to HSPGs. Cleaved Ths might be freed from such glycosylation-mediated "inhibition" and allowed to diffuse farther and faster. The full-length and fully modified form may be protected from proteolysis by glycosylation (VAN DEN STEEN *et al.* 1998), resulting in local FGF signaling, which may be preferred in some cases. Future studies will explore whether Ths and Pyr have different diffusion rates, and if these rates are affected by post-translational modification.

Furthermore, the gradient formed by the HA-Ths-Myc construct may be dependent on the uptake of ligand in a "source-sink" mechanism similar to what is observed for FGF8 diffusion in the zebrafish developing midbrain-hindbrain region (YU *et al.* 2009b). In this scenario, cleavage could produce a form of Ths that is recognized and endocytosed, and may explain the more long-range activity associated with HA-Ths$^{\Delta 261\text{-}356}$-Myc. Along these

lines, short Ths^{1-158} may be lacking such an internalization sequence to support the "flattened" output profile observed.

In the embryo, the switch between secretion of truncated or full-length ligand could be tissue-specific or temporally regulated as a means to support differential activity/range of the ligands. Once the proteases that process Ths and Pyr are uncovered, it will be possible to study the relationship between proteolysis and range-of-action.

Implications for vertebrate studies

Lastly, these new molecular data on Ths and Pyr raise questions about the evolutionary history of the FGF family. All 24 FGF family members in vertebrates are relatively small proteins. Did Ths derive its long C-terminus in the *Drosophila* lineage independently before it was duplicated to produce Pyr, or was the ancestral FGF a long protein with cleavage sites that were lost in the vertebrate lineage? To obtain some insight into these questions, we can look to FGFs characterized in other animal models (TULIN and SATHOPOULOS 2010). Worms have two FGF ligands, LET-756 and EGL-15. EGL-17 is small and LET-756 is 425 aa, an intermediate size, but not known to be cleaved (BIRNBAUM *et al.* 2005; POPOVICI *et al.* 2006). Additionally, Bnl, the other FGF ligand in *Drosophila*, is approximately the same size as Ths and Pyr (i.e., 84 kD), although it is not more related to them than Ths/Pyr are to FGF8 (POPOVICI *et al.* 2005). Therefore, it seems most likely that the *Drosophila* genome tolerates the lengthening of proteins and has found secondary ways of processing them during post-translational regulation. This theory of differential genome tolerance was also put forth by Schmid and Tautz regarding

Drosophila transcription factors, which are on average 30% longer than their *Tribolium* homologs (SCHMID and TAUTZ 1999). Another possibility (which is not mutually exclusive) is that the *Drosophila* FGF ligands are multi-functional proteins, with the FGF-homologous portions responsible for activation of FGFRs and with the low-complexity regions (i.e., C-termini for Pyr and Ths) supporting additional functions, other than receptor-binding, required to support FGF signaling. Furthermore, while Ths and Pyr likely arose from an ancient duplication, (STATHOPOULOS *et al.* 2004), the C-termini of these proteins have diverged: the Pyr C-terminus is most similar to an intracellular protein (i.e., Tncr15 which interacts with the adaptor Grb10) and the Ths C-terminus exhibits homology to highly glycosylated proteins, likely found extracellularly. In vertebrates, studies on the Klotho protein suggest that at least some endocrine FGFs interact with additional proteins to influence receptor binding and activity (URAKAWA *et al.* 2006). Perhaps the *Drosophila* FGFs are ancestral multi-functional ligands that combine ligand-binding and Klotho-like adaptor or HSPG functions. In any case, whether these "long" FGFs are novel inventions of *Drosophilids* or ancient remnants of more ancestral FGFs, we contend that the modular nature of *Drosophila* FGFs may provide important insights into mechanisms that affect FGF activity, which is best examined by comparing the activities of the diverged ligands, Ths and Pyr.

Conclusions

In the present study we have provided evidence for the proteolytic processing of Drosophila FGF ligands Ths and Pyr in both S2 cell culture and the embryo. Functional data was presented showing that truncated, FGF-domain-containing N-termini are capable

of functioning in the embryo without their respective C-termini. Restricted ectopic expression *in vivo* demonstrated the difference in signaling capability between Ths and Pyr in embryos and domain-swapped chimeras highlighted the differences in the C-terminal domains of Ths and Pyr. These findings advance our understanding of the mechanism of FGF signaling in *Drosophila* and also suggest FGF signaling in *Drosophila* may be even more similar to that in vertebrates.

Methods

Prediction Programs

N-glycosylation sites in the *Drosophila* FGFs were predicted using the NetNGlyc Server version 1.0 at http://www.cbs.dtu.dk/services/NetNGlyc/ (BLOM *et al.* 2004). O-glycosylation sites were predicted using OGPET version 1.0 prediction tool, © University of Texas at El Paso (UTEP) El Paso, TX accessed at http://ogpet.utep.edu/OGPET/contact.php.

Fly Stocks and Constructs

69B-GAL4 (Brand and Perrimon, 1993) and zenVRE.Kr-GAL4 (Frasch, 1995) fly stocks have been previously described. All Ths and Pyr truncation, deletion and chimera constructs were inserted into the pUASt-attB vector. 1X HA tags were inserted by fusion PCR just after the N-terminal signal peptide, between amino acid 22 and 23 for Ths (i.e., ALCTV – HAtag – EDYVI) and between amino acid 30 and 31 for Pyr (i.e., ASAAK – HAtag – NVLTL). 6X Myc tags were inserted with Xho1 sites at the C-terminus just

before the stop codon. HA-Ths$^{(1-158)}$, HA-Ths$^{(1-261)}$, HA-Ths$^{(1-403)}$, HA-Pyr$^{(1-220)}$, HA-Pyr$^{(1-348)}$, HA-Pyr$^{(1-466)}$ were all PCR amplified from full *length ths or pyr* template and cloned into pUASt-attB with Not1/Kpn1 sites (Ths constructs) or BglII/Xba (Pyr constructs) sites. Of note is the fact that a full-length *pyramus* cDNA has not been isolated to date, neither from cDNA libraries nor when primers are utilized to PCR amplify the predicted full-length gene from cDNA directly. Therefore, the *pyr* coding sequence in hand is a recombinant DNA molecule composed of 1 kB of cDNA sequence fused to ~1.3 kB of genomic sequence, based on the current genome prediction (KADAM *et al.* 2009).

S2 cell culture and transient transfection

Schneider cells (S2) obtained from the Drosophila Genomics Resource Center (DGRC) were maintained in a 25°C incubator in Schneider's Drosophila Medium (Invitrogen, #11720-067), supplemented with 10% Fetal Bovine Serum (USA Scientific, #9871-5200), Pencillin-streptamycin (dil 1:100), and Fungizone (Invitrogen, #15290018), and filter sterilized. Cells were passed with a 1:10 dilution every 4-5 days.

Effectene transfection reagent (Qiagen, #301425) was used to transiently transfect DNA into S2 cells. 10ul Effectene reagent and 3.4ul Enhancer were used with 1ug DNA in 100ul EC Buffer. Cells were seeded into 6-well culture dishes at a concentration of 2 x 10^6 cells/well. 100uM CuSO4 was added the day after transfection to induce the expression of the vectors from the metallothionine promoter. Supernatant and cell pellet fractions were harvested 2 days post transfection. Cells were lysed with a denaturing lysis buffer (1%

SDS, 50mM Tris, 5mM EDTA, DTT, DNase and protease inhibitor cocktail). Complete Protease Inhibitor Cocktail (Roche) was added to the supernatant fractions.

Immunoprecipitation and Western Blotting

Avidin-conjugated beads from Pierce were used to pull down HA and Myc tagged FGF constructs with a HA-biotin or Myc-biotin antibody. 250ul of supernatant or cell pellet was combined with 50ul of avidin agarose beads (Pierce #20219), 25ul 10x RIPA buffer (500mM Tris pH8, 1.5M NaCl, 5% DOC, 1% SDS, 1-% NP-40, 10mM DTT, 10X Roche Complete protease inhibitors, pH to 8.0) and 0.5µg anti-HA-Biotin (rat, 3F10, Roche #12158167001) or .01ug anti-c-Myc biotin conjugated antibody (mouse, 9E10, Sigma # B7554) and rocked overnight at 4^{o}C. Beads were washed 3 times 5mins with 1X RIPA buffer, and proteins were released from the beads by boiling in 1x SDS sample buffer for 5mins.

Immunoprecipitation samples were run on 10% SDS-PAGE gels, transferred for 6mins with iblot (Invitrogen) and blocked for 1 hour at room temperature in 4% milk in TBS-T. Primary antibodies were used at the following dilutions: 1:1,000 anti-HA (mouse monoclonal, Covance, 16B12) or 1:10,000 anti-Myc (rabbit, AbCam 9106) 1 hour at room temperature. Blots were then washed 3 times 15mins in 1X TBS-T. Secondary antibodies conjugated to HRP were anti-mouse (Upstate, 1 hr. RT, 1:10,000) and anti-rabbit (Biorad, 20mins, 1:10,000). Blots were washed again 3 times 15mins in 1X TBS-T, and once for 5mins in H2O to remove Tween. Visualizer (Upstate) was used to develop the blots.

Generating Site-Directed Transgenics and UAS-GAL4 mediated expression

Ths and Pyr constructs were cloned into the pUASt-attB vector (BISCHOF *et al.* 2007). Proper folding/secretion was assayed in S2 cell culture and by Western Blot before injection. Constructs were injected by site-directed transgenesis into the 86FB location on the third chromosome (BISCHOF *et al.* 2007); Rainbow Transgenics (Newbury Park, CA) performed most injections, some were done in-house.

Immunostaining S2 cells

No. 1 ½, 22mm x 22mm glass coverslips (Corning, #2870-22) were cleaned by soaking in HCl for 1hr. and rinsed thoroughly with dH_2O. The coverslips were air dried and treated with 50uL of 1mg/mL concanavalin A (MP Biomedicals, #195283). Transiently transfected S2 cells were allowed to spread on the coverslips and attach to the ConA coating. The cells were fixed in 4% paraformaldehyde for 15mins, rinsed 3X with PBT (1X PBS + 1% Triton), and blocked in 5% Normal Goat Serum (Invitrogen) for 10mins. Primary antibodies were added in 5% block for 1 hr at RT, and then rinsed off. Antibodies used were: rabbit anti-GFP (1:1000, Invitrogen, #A111-22), mouse anti-HA (1:1000, Covance #16B12) and rabbit anti-Myc (1:10,000, AbCam #9106). Secondary antibodies (anti-rabbit 555, 1:500, Alexa Fluor, Invitrogen) were also added in 5% block for 1 hr at RT and rinsed off. Triton was rinsed off with two washes in H_2O. Samples were mounted to slides with Vectashield Hardmount.

Immunostaining Drosophila Embryos

3-6 hr embryos were collected and dechorinated in 50% Bleach for 3mins. The embryos were fixed in Heptane Fixing Solution [0.4mL formaldehyde, 4ml Heptane, 3.6mL Fixing Buffer (10mM KPO4, pH6.8, 15mM NaCl, 45mM KCl, 2mM MgCl2)] for 12mins on an orbital shaker on high. The Heptane/Formaldehyde was removed and replaced with MeOH. The embryos were rinsed in MeOH 4 times and stored at -20^{o}C for short term or -80^{o}C for long term. Embryos were blocked in 1x western blocking reagent (Roche) for 30mins RT, and primary antibody incubations were performed overnight. Primary antibodies used were: anti-Even skipped rabbit (1:1000, M. Frasch) and anti-βgal rabbit (1:250, Molecular Probes). Secondary antibody was applied for 1-2 hrs. at RT: anti-rabbit 1:200 (Vectastain, Vector labs).

***In vitro* transcription/translation**

TnT T7/T3 Coupled Reticulocyte Lysate System (Promega, #L5010) was used with pBS-Pyr and pBS-Ths, incorporating S^{35} Methionine, to assay the unmodified, full-length size of the proteins. Transcend^{TM} Non-radioactive Translation Detection System (Promega, #L5070) containing biotinylated lysine in the Transcend tRNA was used in conjunction with the TnT Coupled Reticulocyte kit to transcribe and translate pUASt-HA-Pyr$^{(1-348)}$ and pUASt-HA-Pyr$^{(1-466)}$, which were subsequently run on 10% SDS-PAGE and detected with anti-HA by Western Blot.

Chapter 4

[part of this chapter was published in Birth Defects Research Part C: Embryo Today as:
Tulin S, Stathopoulos A, 2010. Extending the family table: Insights from beyond vertebrates into the regulation of embryonic development by FGFs. Birth Defects Research Part C: Embryo Today 90: 214-27.]

EVOLUTIONARY PERSPECTIVE OF THE FGF SUPERFAMILY

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 models to each other is diagrammed 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) (GRYZIK and MÜLLER 2004a; STATHOPOULOS *et al.* 2004). 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 anthozoan 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).

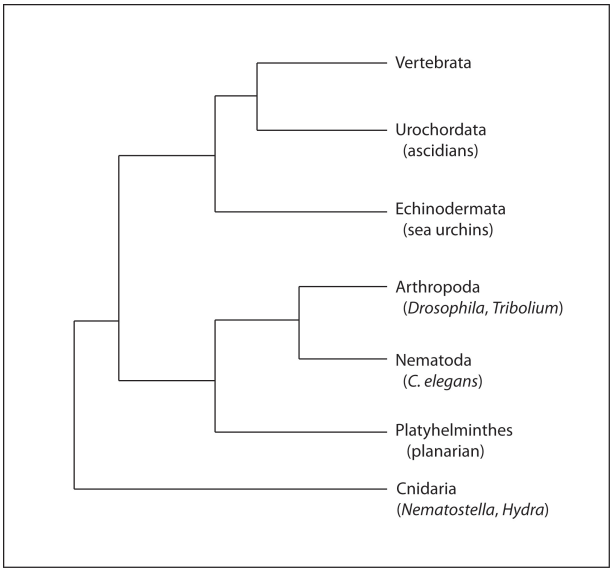

Figure 1 | Simplified tree of metazoan phyla.

Simplified tree of phyla with FGF family representatives discussed in the text. Branch lengths are not to scale.

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 homologous transcripts containing FGF domains were found in the *Nematostella* genome, but so far only 4 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 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 haven't 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 4 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. 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*. 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 in nonvertebrate models of the mechanism of Sprouty regulation may aid the advancement of its use in therapies for certain diseases.

Regulation has also been found to come from co-expressed FGF ligands. 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 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

Surveying FGF genes from across metazoa provides several points on the map of evolution of FGF signaling. 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 FGFs and 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

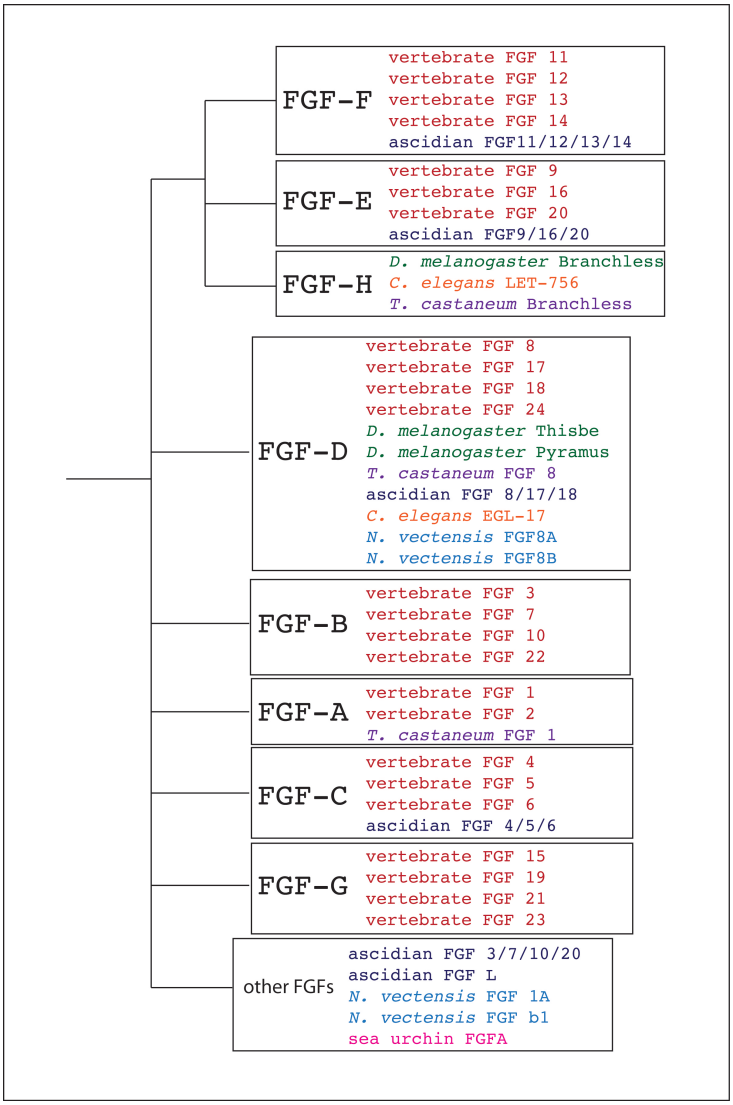

Figure 2 | Phylogenetic relationships of FGF family members

Relationships displayed according to the previously described eight group classification (POPOVICI *et al.* 2005). The simplified tree and the positions of FGF family members are based on Fig. 1 in Matus et al. (MATUS *et al.* 2007). Positions of *Tribolium* FGF genes are based on the analysis by Beerman and Schröder (BEERMANN and SCHRÖDER 2008).

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) (Figure 2). *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 was 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,* and *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 cardiomyocytes 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 some kind of function in heart development (although possibly not homologous) compared to FGF9/16/20 in *Ciona*. FGF9 and FGF16 seem to be still redundant 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. 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 between *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.

Table 1

	aa	kD	known functions	expressed in	references
C. intestinalis					
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
D. melanogaster					
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
T. Castaneum					
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	primary vuval cell	Burdine et. al, 1997; Burdine et. al, 1998
			essential, regulation of fluid balance, axonal migration, negative	secreted and nuclear, muscles adjacent to hypodermis	Roubin et. al, 1999; Popovici et. al, 2006; Dixon et. al, 2006
LET-756	425	50	regulation of muscle membrane		
N. vectensis					
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

Table 1 | Survey of described invertebrate FGF ligands

Summary of information available for non-vertebrate FGFs, with selected references. *C. intestinalis* (sea squirt), *S. purpuratus* (sea urchin), *D. Melanogaster* (fruit fly), *T. castaneum* (flour beetle), *C. elegans* (nematode worm), *N. vectensis* (sea anemone).

Drosophila vs. 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) to be expressed (BEERMANN and SCHRÖDER 2008). *Tribolium* and *Drosophila* are more than 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. 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*. *Pyr* and *ths* both 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.* 2009b). 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 to be expressed in largely the same domains as *pyr/ths* during embryogenesis and so likely also 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 neighbouring 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.* FGF1 is ubiquitously expressed and is known to play a developmental and maintenance role of neuronal tissue. 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 an 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 unconserved domains outside the FGF domain has received little attention in most FGFs. Two notable exceptions are the study of FGF9/FGF20 and FGF23 in vertebrates.

The crystal structures of FGF9 and FGFF20 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 dimer, 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* in *Drosophila* (TULIN and STATHOPOULOS 2010). 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 might play a role in the rate of ligand diffusion and/or potency by an unknown mechanism. 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. The role of FGF processing in the embryo and its requirements for function are yet to be fully understood.

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 birth with a hypoplastic diaphragm 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 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 are 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 soluble state with high affinity. The affinity of FGFRL1 for FGF3 is 1 order of magnitude 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*, which has an 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 to in all of the discussed areas and will likely provide targets for medical applications. Importantly, FGF signaling is an ancient cell communication mechanism that has been utilized by animals at least since cnidarians first appeared, and is present in all the major modern surveyed phyla. 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.

Chapter 5

DISCUSSION AND FUTURE DIRECTIONS

Summary

The aim of my thesis research was to understand the regulation of Fibroblast Growth Factor signaling using *Drosophila melanogastor* as a model system and investigating the details of Thisbe and Pyramus FGF ligands activating the Heartless FGF receptor. Previous work from our group indicated that there might be a difference in the diffusion range or binding capabilities of Ths as compared to Pyr (KADAM *et al.* 2009; STATHOPOULOS *et al.* 2004), and these properties could contribute to differential regulation of the ligands. To evaluate this possibility we undertook an extensive analysis of the functional domains of both Ths and Pyr using both *Drosophila* cell culture and *Drosophila* embryos. We demonstrated for the first time that in S2 cells Ths and Pyr are not secreted at their predicted size but are detected as smaller forms, indicating they are cleaved in cell culture. We also found cleaved forms present in embryonic extracts, demonstrating that cleavage may take place during normal development in the embryo as well. This result was crucial because it demonstrates the relevance of the information obtained in cell culture to the assays performed in the embryo.

To dissect the function of the N- and C-terminal domains of Ths and Pyr, we created a set of truncation, chimera and deletion constructs. The cleaved forms of Ths and Pyr in cell culture contain the FGF core domain and suggest the possibility that the N-terminal

domain is capable of signaling without the C-terminus. We used the truncated constructs to show that indeed the N-terminus of both Ths and Pyr containing the FGF core domain is able to function without the C-terminus. We then asked if there was any function for the unconserved domain at the C-terminus of Ths and Pyr. Using Thisbe-Pyramus domain swapped chimeric constructs we were able to see that the C-terminus of Ths appears to be inhibitory while the C-terminus of Pyr is not. The reason for the functional difference may be due to the differential subcellular localization of the C-termini of Ths and Pyr. While the C-terminus of Ths is detectable outside of the cell and appears to be secreted as part of full-length Ths constructs and possibly also as C-terminal cleaved piece, the C-terminus of Pyr is retained within the cell, inside an organelle that may be lysosomal in character.

Whether cleavage is a necessary step for some or all functions of Ths and Pyr is yet to be determined. We engineered a Ths construct with the region containing putative cleavage sites deleted, and tested it in the same assays as the other truncation and chimera constructs. Although there was a shift to more full-length product being secreted, some cleaved forms were still made. In the embryo, the construct was still functional, leaving open the possibility that either the remaining cleaved forms are sufficient for function or that cleavage is not required for the particular function we were assaying (differentiation of mesodermal cells into Eve-expressing pericardial cells). There are several other described functions for Ths and Pyr in the developing embryo: mesoderm migration, caudal visceral mesoderm migration and axonal migration/glial wrapping. It would be interesting to assay the cleavage-compromised

construct in the context of these other functions. To globally assay if cleavage is required for any function in the embryo rescue constructs of Thisbe and Pyramus could be engineered with a deletion of the cleavage sites.

Future Directions

My work was the first to show proteolytic processing of Ths and Pyr ligands, and the first case of processing among FGF ligands in a developmental context (FGF23 function being limited to homeostatic regulation). Proteolytic processing is a very common mechanism to regulate the activity or activation of signaling ligands and has already been described in detail for other ligands in vertebrates and *Drosophila,* as discussed in Chapter 2. My thesis work has formed a foundation for understanding the regulation of Ths and Pyr proteins through processing and understanding how regulation is related to functional specificity in a system with multiple ligands using the same receptor (which is always the case in vertebrates). The results of my thesis research have opened several new paths of questioning. Most importantly, "Is cleavage actively regulating the activity of Ths and Pyr and contributing to their developmental functions?" This question can be addressed by using genomic rescue constructs with cleavage sites deleted for Ths and Pyr and testing them in *Drosophila* developmental contexts. For instance, perhaps cleavage is regulative during mesoderm spreading and intercalation. We can use Ths and Pyr single mutant backgrounds and supply only the deleted construct to see if mesoderm spreading is altered (single mutants described in (KADAM *et al.* 2009; KLINGSEISEN *et al.* 2009a)).

Another question to address would be, "Is Branchless also cleaved and processed in a manner similar to Ths and/or Pyr?" Bnl, at 770aa, is predicted to be about the same size as Ths and Pyr (SUTHERLAND *et al.* 1996). However, instead of its FGF core domain following immediately after the N-terminal secretion signal, Bnl has both N-terminal and C-terminal tails with the FGF core domain closer to the middle (See Chapter 3, Figure 1). There are predicted cleavage sites both on the N-terminal side of the FGF domain and in the C-terminus (inverted white triangles, Fig. 1, Ch. 3). A similar analysis as we have performed for Ths and Pyr should be followed for Bnl to determine if cleavage is a common mechanism among all known *Drosophila* FGFs. Epitope tagging both the N- and C-terminal end of Bnl and transiently transfecting it into S2 cells and immunoprecipitating from the supernatant will reveal if similar cleavage mechanisms are working on Bnl. In the embryo, Bnl is responsible for branch outgrowth in the developing trachea. Overexpression phenotypes have already been documented for UAS-Bnl using C49-GAL4 (new, ectopic branches) and could be used to analyze different truncation constructs (SUTHERLAND *et al.* 1996).

The processing events of Ths and Pyr should be further characterized. We would first use S2 cells to screen known proteases in *Drosophila* for their ability to cleave Ths and Pyr. Chemicals are available to inhibit specific classes of proteases to narrow down which class contains the responsible protease(s) in a manner similar to that of Urban et. al (URBAN *et al.* 2001). Serine proteases are inhibited by 3,4-dichloroisocoumarin (DCI), and tosyl phenylalanine chloromethyl ketone (TPCK). Cysteine proteases are sensitive to E64d and leupeptin (SALVESEN 2001); whereas calpain proteases are sensitive to PD150606 (WANG *et al.* 1996). Aspartyl proteases are sensitive to the

inhibitor pepstatin A and presenilin proteases are inhibited by γ–secretase (HARTMANN *et al.* 1997). Metalloproteases are inhibited by batimastat and ilomastat (LEE *et al.* 2001). Finally, Furin proteases are inhibited by Dec-RVKR-CMK (STIENEKE-GRÖBER *et al.* 1992) and this is the protease inhibitor we will test first because of the putative Furin-like proprotein convertase sites present in Pyr, Ths and Bnl. Additional information about the cleavage of Ths and Pyr can be obtained by making smaller deletions and even single site mutations to determine the order of cleavage events and the identity of the sites that are recognized. In the case of FGF23, identification of the site of cleavage was aided by the availability of human mutations present that disrupted the cleavage site, however the exact identities of the protease(s) responsible are still not known (BENET-PAGÈS *et al.* 2004). To clearly define the cleavage mechanism, the responsible protease(s), their site(s) of cleavage and the order of cleaved sites all need to be specified.

The aspect of Ths and Pyr signaling that we are most interested in is how the properties of the ligands relate to the function FGF signaling in the Drosophila embryo. To fully understand the signaling abilities of both ligands, it is imperative that the binding kinetics of Ths and Pyr to the Heartless receptor are described in detail. Crystallography has already been performed for a number of FGFs including FGF1, FGF2, FGF9 and FGF20 (PLOTNIKOV *et al.* 2001). The crystal structures were required to understand the amino acids involved in binding to HSPGs, the receptor, and in the case of FGF9 subfamily members, the regions involved in homodimerization. No crystal structures have yet been determined for any non-vertebrate FGFs and this level

of analysis would aid in better comparisons of FGF between species and to understand mechanisms. There is some evidence that HSPGs may be involved in FGF signaling in *Drosophila*, but it is not as well supported or documented as for vertebrates. *Drosophila* HSPG mutants for the glypican *Dally-like* and HSPG modifying enzymes *sugarless* and *sulfateless* all show defects in either *heartless* or *breathless*-mediated signaling (LIN and PERRIMON 2002; YAN and LIN 2007). Further studies on HSPGs in *Drosophila* are needed to understand their role and how it compares to the situation in vertebrates. Crystallography with and without HSPGs for Pyr, Ths and Bnl would be valuable information for the field.

More specific kinetic analysis for FGF binding could be obtained by using Biacore surface plasmon resonance based technology. In this system either the ligands or receptors are fixed to chips through engineered immunological domains and the corresponding ligands or receptors are then washed over the surface while binding strength and duration is calculated. We may find that Pyr binds more strongly to Htl but dissociates more quickly that Ths or vice versa. These results could then be applied to our knowledge of the requirements for Ths and Pyr during developmental processes. An additional assay that would be helpful for this analysis would be to develop a cell culture assay in S2 cells where binding of the ligands to the receptor can be quantitatively measured, as they have done in vertebrate cells to compare the binding strength for different ligand/receptor combinations (ORNITZ *et al.* 1996; ZHANG *et al.* 2006). One barrier to developing this assay is the dependence of Htl signaling on the co-factor Stumps (as discussed on page 26-27). Stumps will likely have to be

permanently transfected along with Htl in order to establish a cell line that will respond to FGF ligands. The response could likely be measured by di-phosphorylated MAPK production. This assay would be useful for comparing the response of both Btl and Htl to Bnl, Ths and Pyr.

An aspect of FGF signaling that I proposed to work on, but did not get a chance to address during my thesis work, is the role of FGF signaling in the regulatory network of *Drosophila* development. An early promoter for *ths* has been found, but lacks a complete analysis. It is known that *ths* is turned on in the neurogenic ectoderm by the Dorsal transcription factor as part of a suite of targets to pattern the early dorsal/ventral axis (STATHOPOULOS *et al.* 2004). Additionally, it is known that there are multiple combinations of transcription factor sites that can produce a similar stripe of expression in the neurogenic ectoderm (LIBERMAN and STATHOPOULOS 2009). The *pyr* enhancer(s) have not yet been described. Understanding how the FGF ligands are activated and repressed at the level of *cis*-regulation is a key piece of the FGF signaling puzzle. In order to address this, we need to make genomic constructs that recapitulate all of the expression of *ths* and *pyr* in the embryo. There will likely be multiple enhancers for the different temporal and spatial regions of FGF expression. Additionally, the *cis*-regulatory analysis is likely to be a very rich study because the expression of *ths* and *pyr* is highly dynamic and likely requires the coordinated action of many activators and repressors. The downstream targets of FGF signaling also need to be identified. In order to understand if a different battery of genes is turned on in response to signaling by *ths* as compared to signaling by *pyr* or *bnl*, a microarray

approach could be used along with single mutants for *ths* and *pyr*. A study of genes turned on in response to Btl signaling has already been published (STAHL *et al.* 2007). It will be interesting to compare the downstream targets turned on for different functions to see the similarities and differences.

Another mechanism discussed in Chapter 4 for generating FGF signaling specificity is the generation of multiple spliceforms of either FGF ligands or receptors. It is currently thought that Htl exists in only one splice form; however, a careful analysis has not been undertaken to say this with certainty. To understand the complete picture of FGF regulation, this possibility must be taken into consideration and tested.

The continuation of work in this area will necessarily take place at the intersection of functional genetics, cis-regulatory analysis, and enzyme biochemistry. All the tools available in *Drosophila* make the complete analysis of FGF signaling and regulation in the fly a realistic possibility and the likely gains to the FGF field as a whole are considerable.

BIBLIOGRAPHY

AHMAD, S. M., and B. S. BAKER, 2002 Sex-specific deployment of FGF signaling in Drosophila recruits mesodermal cells into the male genital imaginal disc. Cell **109:** 651-661.

AMY MCMAHON, G. R., WILLY SUPATTO AND ANGELIKE STATHOPOULOS, 2010 Mesoderm migration in Drosophila is a multi-step process requiring FGF signaling and integrin activity. Development **137:** in press.

ANDERSON, E. D., J. K. VANSLYKE, C. D. THULIN, F. JEAN and G. THOMAS, 1997 Activation of the furin endoprotease is a multiple-step process: requirements for acidification and internal propeptide cleavage. EMBO J **16:** 1508-1518.

AONO, A., M. HAZAMA, K. NOTOYA, S. TAKETOMI, H. YAMASAKI *et al.*, 1995 Potent ectopic bone-inducing activity of bone morphogenetic protein-4/7 heterodimer. Biochem Biophys Res Commun **210:** 670-677.

ARMELIN, H. A., 1973 Pituitary extracts and steroid hormones in the control of 3T3 cell growth. Proc Natl Acad Sci USA **70:** 2702-2706.

BAERTSCHI, S., L. ZHUANG and B. TRUEB, 2007 Mice with a targeted disruption of the Fgfrl1 gene die at birth due to alterations in the diaphragm. FEBS J **274:** 6241-6253.

BAI, X., D. MIAO, J. LI, D. GOLTZMAN and A. C. KARAPLIS, 2004 Transgenic mice overexpressing human fibroblast growth factor 23 (R176Q) delineate a putative role for parathyroid hormone in renal phosphate wasting disorders. Endocrinology **145:** 5269-5279.

BAI, X. Y., D. MIAO, D. GOLTZMAN and A. C. KARAPLIS, 2003 The autosomal dominant hypophosphatemic rickets R176Q mutation in fibroblast growth factor 23 resists proteolytic cleavage and enhances in vivo biological potency. J Biol Chem **278:** 9843-9849.

BEENKEN, A., and M. MOHAMMADI, 2009 The FGF family: biology, pathophysiology and therapy. Nature reviews Drug discovery **8:** 235-253.

BEERMANN, A., and R. SCHRÖDER, 2008 Sites of Fgf signalling and perception during embryogenesis of the beetle Tribolium castaneum. Dev Genes Evol **218:** 153-167.

BEH, J., W. SHI, M. LEVINE, B. DAVIDSON and L. CHRISTIAEN, 2007 FoxF is essential for FGF-induced migration of heart progenitor cells in the ascidian Ciona intestinalis. Development **134:** 3297-3305.

BEIMAN, M., B. Z. SHILO and T. VOLK, 1996 Heartless, a Drosophila FGF receptor homolog, is essential for cell migration and establishment of several mesodermal lineages. Genes Dev **10:** 2993-3002.

BEN-HAIM, N., C. LU, M. GUZMAN-AYALA, L. PESCATORE, D. MESNARD *et al.*, 2006 The nodal precursor acting via activin receptors induces mesoderm by maintaining a source of its convertases and BMP4. Dev Cell **11:** 313-323.

BENET-PAGÈS, A., B. LORENZ-DEPIEREUX, H. ZISCHKA, K. E. WHITE, M. J. ECONS *et al.*, 2004 FGF23 is processed by proprotein convertases but not by PHEX. Bone **35:** 455-462.

BERTRAND, S., I. SOMORJAI, J. GARCIA-FERNANDEZ, T. LAMONERIE and H. ESCRIVA, 2009 FGFRL1 is a neglected putative actor of the FGF signalling pathway present in all major metazoan phyla. BMC Evol Biol **9:** 226.

BERTRAND, V., C. HUDSON, D. CAILLOL, C. POPOVICI and P. LEMAIRE, 2003 Neural tissue in ascidian embryos is induced by FGF9/16/20, acting via a combination of maternal GATA and Ets transcription factors. Cell **115:** 615-627.

BEYER, T. A., S. WERNER, C. DICKSON and R. GROSE, 2003 Fibroblast growth factor 22 and its potential role during skin development and repair. Exp Cell Res **287:** 228-236.

BIKFALVI, A., S. KLEIN, G. PINTUCCI and D. B. RIFKIN, 1997 Biological roles of fibroblast growth factor-2. Endocr Rev **18:** 26-45.

BIRNBAUM, D., C. POPOVICI and R. ROUBIN, 2005 A pair as a minimum: the two fibroblast growth factors of the nematode Caenorhabditis elegans. Dev Dyn **232:** 247-255.

BISCHOF, J., R. K. MAEDA, M. HEDIGER, F. KARCH and K. BASLER, 2007 An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. Proc Natl Acad Sci USA **104:** 3312-3317.

BLAND, C. E., P. KIMBERLY and M. D. RAND, 2003 Notch-induced proteolysis and nuclear localization of the Delta ligand. J Biol Chem **278:** 13607-13610.

BLAUMUELLER, C. M., H. QI, P. ZAGOURAS and S. ARTAVANIS-TSAKONAS, 1997 Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. Cell **90:** 281-291.

BLOM, N., T. SICHERITZ-PONTÉN, R. GUPTA, S. GAMMELTOFT and S. BRUNAK, 2004 Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. Proteomics **4:** 1633-1649.

BÖTTCHER, R. T., and C. NIEHRS, 2005 Fibroblast growth factor signaling during early vertebrate development. Endocr Rev **26:** 63-77.

BRIDGE, D., C. W. CUNNINGHAM, R. DESALLE and L. W. BUSS, 1995 Class-level relationships in the phylum Cnidaria: molecular and morphological evidence. Mol Biol Evol **12:** 679-689.

BRIDGE, D., C. W. CUNNINGHAM, B. SCHIERWATER, R. DESALLE and L. W. BUSS, 1992 Class-level relationships in the phylum Cnidaria: evidence from mitochondrial genome structure. Proc Natl Acad Sci USA **89:** 8750-8753.

BROU, C., F. LOGEAT, N. GUPTA, C. BESSIA, O. LEBAIL *et al.*, 2000 A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. Mol Cell **5:** 207-216.

BUFF, E., A. CARMENA, S. GISSELBRECHT, F. JIMÉNEZ and A. M. MICHELSON, 1998 Signalling by the Drosophila epidermal growth factor receptor is required for the specification and diversification of embryonic muscle progenitors. Development **125:** 2075-2086.

BURDINE, R. D., C. S. BRANDA and M. J. STERN, 1998 EGL-17(FGF) expression coordinates the attraction of the migrating sex myoblasts with vulval induction in C. elegans. Development **125:** 1083-1093.

BURDINE, R. D., E. B. CHEN, S. F. KWOK and M. J. STERN, 1997 egl-17 encodes an invertebrate fibroblast growth factor family member required specifically for

sex myoblast migration in Caenorhabditis elegans. Proc Natl Acad Sci USA **94:** 2433-2437.

BURGESS, W. H., and T. MACIAG, 1989 The heparin-binding (fibroblast) growth factor family of proteins. Annu Rev Biochem **58:** 575-606.

BURGESS, W. H., T. MEHLMAN, D. R. MARSHAK, B. A. FRASER and T. MACIAG, 1986 Structural evidence that endothelial cell growth factor beta is the precursor of both endothelial cell growth factor alpha and acidic fibroblast growth factor. Proc Natl Acad Sci USA **83:** 7216-7220.

CAPPELLEN, D., C. DE OLIVEIRA, D. RICOL, S. DE MEDINA, J. BOURDIN *et al.*, 1999 Frequent activating mutations of FGFR3 in human bladder and cervix carcinomas. Nat Genet **23:** 18-20.

CARMENA, A., M. BATE and F. JIMÉNEZ, 1995 Lethal of scute, a proneural gene, participates in the specification of muscle progenitors during Drosophila embryogenesis. Genes Dev **9:** 2373-2383.

CARMENA, A., S. GISSELBRECHT, J. HARRISON, F. JIMÉNEZ and A. M. MICHELSON, 1998a Combinatorial signaling codes for the progressive determination of cell fates in the Drosophila embryonic mesoderm. Genes Dev **12:** 3910-3922.

CARMENA, A., B. MURUGASU-OEI, D. MENON, F. JIMÉNEZ and W. CHIA, 1998b Inscuteable and numb mediate asymmetric muscle progenitor cell divisions during Drosophila myogenesis. Genes Dev **12:** 304-315.

CASCI, T., J. VINÓS and M. FREEMAN, 1999 Sprouty, an intracellular inhibitor of Ras signaling. Cell **96:** 655-665.

CATELA, C., D. BILBAO-CORTES, E. SLONIMSKY, P. KRATSIOS, N. ROSENTHAL *et al.*, 2009 Multiple congenital malformations of Wolf-Hirschhorn syndrome are recapitulated in Fgfrl1 null mice. Dis Model Mech **2:** 283-294.

CEBRIÀ, F., C. KOBAYASHI, Y. UMESONO, M. NAKAZAWA, K. MINETA *et al.*, 2002 FGFR-related gene nou-darake restricts brain tissues to the head region of planarians. Nature **419:** 620-624.

CHASAN, R., and K. V. ANDERSON, 1989 The role of easter, an apparent serine protease, in organizing the dorsal-ventral pattern of the Drosophila embryo. Cell **56:** 391-400.

CHEN, L., and C. X. DENG, 2005 Roles of FGF signaling in skeletal development and human genetic diseases. Front Biosci **10:** 1961-1976.

CHI, C. L., S. MARTINEZ, W. WURST and G. R. MARTIN, 2003 The isthmic organizer signal FGF8 is required for cell survival in the prospective midbrain and cerebellum. Development **130:** 2633-2644.

CHOURROUT, D., F. DELSUC, P. CHOURROUT, R. B. EDVARDSEN, F. RENTZSCH *et al.*, 2006 Minimal ProtoHox cluster inferred from bilaterian and cnidarian Hox complements. Nature **442:** 684-687.

CIRUNA, B., and J. ROSSANT, 2001 FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. Dev Cell **1:** 37-49.

CIRUNA, B. G., L. SCHWARTZ, K. HARPAL, T. P. YAMAGUCHI and J. ROSSANT, 1997 Chimeric analysis of fibroblast growth factor receptor-1 (Fgfr1) function: a role for FGFR1 in morphogenetic movement through the primitive streak. Development **124:** 2829-2841.

COFFIN, J. D., R. Z. FLORKIEWICZ, J. NEUMANN, T. MORT-HOPKINS, G. W. DORN *et al.*, 1995 Abnormal bone growth and selective translational regulation in basic fibroblast growth factor (FGF-2) transgenic mice. Mol Biol Cell **6:** 1861-1873.

COLEMAN-KRNACIK, S., and J. M. ROSEN, 1994 Differential temporal and spatial gene expression of fibroblast growth factor family members during mouse mammary gland development. Mol Endocrinol **8:** 218-229.

COLLINS, A. G., P. SCHUCHERT, A. C. MARQUES, T. JANKOWSKI, M. MEDINA *et al.*, 2006 Medusozoan phylogeny and character evolution clarified by new large and small subunit rDNA data and an assessment of the utility of phylogenetic mixture models. Syst Biol **55:** 97-115.

COLVIN, J. S., B. A. BOHNE, G. W. HARDING, D. G. MCEWEN and D. M. ORNITZ, 1996 Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. Nat Genet **12:** 390-397.

COLVIN, J. S., R. P. GREEN, J. SCHMAHL, B. CAPEL and D. M. ORNITZ, 2001 Male-to-female sex reversal in mice lacking fibroblast growth factor 9. Cell **104:** 875-889.

CONSTAM, D. B., and E. J. ROBERTSON, 1999 Regulation of bone morphogenetic protein activity by pro domains and proprotein convertases. J Cell Biol **144:** 139-149.

COULIER, F., P. PONTAROTTI, R. ROUBIN, H. HARTUNG, M. GOLDFARB *et al.*, 1997 Of worms and men: an evolutionary perspective on the fibroblast growth factor (FGF) and FGF receptor families. J Mol Evol **44:** 43-56.

COUMOUL, X., and C.-X. DENG, 2003a Roles of FGF receptors in mammalian development and congenital diseases. Birth Defects Res C Embryo Today **69:** 286-304.

COUMOUL, X., and C. X. DENG, 2003b Roles of FGF receptors in mammalian development and congenital diseases. Birth Defects Res C Embryo Today **69:** 286-304.

CROSSLEY, P. H., S. MARTINEZ and G. R. MARTIN, 1996 Midbrain development induced by FGF8 in the chick embryo. Nature **380:** 66-68.

CUI, Y., R. HACKENMILLER, L. BERG, F. JEAN, T. NAKAYAMA *et al.*, 2001 The activity and signaling range of mature BMP-4 is regulated by sequential cleavage at two sites within the prodomain of the precursor. Genes Dev **15:** 2797-2802.

CUI, Y., F. JEAN, G. THOMAS and J. L. CHRISTIAN, 1998 BMP-4 is proteolytically activated by furin and/or PC6 during vertebrate embryonic development. EMBO J **17:** 4735-4743.

DAILEY, L., D. AMBROSETTI, A. MANSUKHANI and C. BASILICO, 2005 Mechanisms underlying differential responses to FGF signaling. Cytokine Growth Factor Reviews **16:** 233-247.

DAVIDSON, B., W. SHI, J. BEH, L. CHRISTIAEN and M. LEVINE, 2006 FGF signaling delineates the cardiac progenitor field in the simple chordate, Ciona intestinalis. Genes Dev **20:** 2728-2738.

DEGNIN, C., F. JEAN, G. THOMAS and J. L. CHRISTIAN, 2004 Cleavages within the prodomain direct intracellular trafficking and degradation of mature bone morphogenetic protein-4. Mol Biol Cell **15:** 5012-5020.

DELAUNE, E., P. LEMAIRE and L. KODJABACHIAN, 2005 Neural induction in Xenopus requires early FGF signalling in addition to BMP inhibition. Development **132:** 299-310.

DELLI BOVI, P., and C. BASILICO, 1987 Isolation of a rearranged human transforming gene following transfection of Kaposi sarcoma DNA. Proc Natl Acad Sci USA **84:** 5660-5664.

DELOTTO, Y., and R. DELOTTO, 1998 Proteolytic processing of the Drosophila Spätzle protein by easter generates a dimeric NGF-like molecule with ventralising activity. Mech Dev **72:** 141-148.

DENG, C., A. WYNSHAW-BORIS, F. ZHOU, A. KUO and P. LEDER, 1996 Fibroblast growth factor receptor 3 is a negative regulator of bone growth. Cell **84:** 911-921.

DENG, C. X., A. WYNSHAW-BORIS, M. M. SHEN, C. DAUGHERTY, D. M. ORNITZ *et al.*, 1994 Murine FGFR-1 is required for early postimplantation growth and axial organization. Genes Dev **8:** 3045-3057.

DICKSON, C., R. SMITH, S. BROOKES and G. PETERS, 1984 Tumorigenesis by mouse mammary tumor virus: proviral activation of a cellular gene in the common integration region int-2. Cell **37:** 529-536.

DIXON, S. J., M. ALEXANDER, R. FERNANDES, N. RICKER and P. J. ROY, 2006 FGF negatively regulates muscle membrane extension in Caenorhabditis elegans. Development **133:** 1263-1275.

DOSSENBACH, C., S. RÖCK and M. AFFOLTER, 2001 Specificity of FGF signaling in cell migration in Drosophila. Development **128:** 4563-4572.

DRAPER, B. W., D. W. STOCK and C. B. KIMMEL, 2003 Zebrafish fgf24 functions with fgf8 to promote posterior mesodermal development. Development **130:** 4639-4654.

DUBOC, V., and M. P. LOGAN, 2009 Building limb morphology through integration of signalling modules. Curr Opin Genet Dev **19:** 497-503.

DUTTA, D., S. SHAW, T. MAQBOOL, H. PANDYA and K. VIJAYRAGHAVAN, 2005 Drosophila Heartless acts with Heartbroken/Dof in muscle founder differentiation. PLoS Biol **3:** e337.

EHEBAUER, M., P. HAYWARD and A. MARTINEZ-ARIAS, 2006 Notch signaling pathway. Sci STKE **2006:** cm7.

ESWARAKUMAR, V. P., I. LAX and J. SCHLESSINGER, 2005 Cellular signaling by fibroblast growth factor receptors. Cytokine Growth Factor Rev **16:** 139-149.

FLETCHER, R. B., J. C. BAKER and R. M. HARLAND, 2006 FGF8 spliceforms mediate early mesoderm and posterior neural tissue formation in Xenopus. Development **133:** 1703-1714.

FORTINI, M. E., 2002 Gamma-secretase-mediated proteolysis in cell-surface-receptor signalling. Nat Rev Mol Cell Biol **3:** 673-684.

FRANZDÓTTIR, S. R., D. ENGELEN, Y. YUVA-AYDEMIR, I. SCHMIDT, A. AHO *et al.*, 2009 Switch in FGF signalling initiates glial differentiation in the Drosophila eye. Nature **460:** 758-761.

FRASCH, M., 1995 Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early Drosophila embryo. Nature **374:** 464-467.

FRASCH, M., and M. LEVINE, 1987 Complementary patterns of even-skipped and fushi tarazu expression involve their differential regulation by a common set of segmentation genes in Drosophila. Genes Dev **1:** 981-995.

FREEMAN, M., 1994 The spitz gene is required for photoreceptor determination in the Drosophila eye where it interacts with the EGF receptor. Mech Dev **48:** 25-33.

FUERER, C., S. J. HABIB and R. NUSSE, 2010 A study on the interactions between heparan sulfate proteoglycans and Wnt proteins. Dev Dyn **239:** 184-190.

FUKUMOTO, S., 2005 Post-translational modification of Fibroblast Growth Factor 23. Ther Apher Dial **9:** 319-322.

GERBER, S. D., F. STEINBERG, M. BEYELER, P. M. VILLIGER and B. TRUEB, 2009 The murine Fgfrl1 receptor is essential for the development of the metanephric kidney. Dev Biol **335:** 106-119.

GILBERT, S., 2006 *Developmental Biology*. Sinauer Associates, Inc., Sunderland, Massachusetts.

GIOVANNONE, B., E. LEE, L. LAVIOLA, F. GIORGINO, K. A. CLEVELAND *et al.*, 2003 Two novel proteins that are linked to insulin-like growth factor (IGF-I) receptors by the Grb10 adapter and modulate IGF-I signaling. J Biol Chem **278:** 31564-31573.

GISSELBRECHT, S., J. B. SKEATH, C. Q. DOE and A. M. MICHELSON, 1996 heartless encodes a fibroblast growth factor receptor (DFR1/DFGF-R2) involved in the directional migration of early mesodermal cells in the Drosophila embryo. Genes Dev **10:** 3003-3017.

GOETZ, R., Y. NAKADA, M. C. HU, H. KUROSU, L. WANG *et al.*, 2010 Isolated C-terminal tail of FGF23 alleviates hypophosphatemia by inhibiting FGF23-FGFR-Klotho complex formation. Proc Natl Acad Sci USA **107:** 407-412.

GOODMAN, S. J., C. S. BRANDA, M. K. ROBINSON, R. D. BURDINE and M. J. STERN, 2003 Alternative splicing affecting a novel domain in the C. elegans EGL-15 FGF receptor confers functional specificity. Development **130:** 3757-3766.

GOSPODAROWICZ, D., 1974 Localisation of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. Nature **249:** 123-127.

GOSPODAROWICZ, D., 1975 Purification of a fibroblast growth factor from bovine pituitary. J Biol Chem **250:** 2515-2520.

GOSPODAROWICZ, D., and J. S. MORAN, 1975 Mitogenic effect of fibroblast growth factor on early passage cultures of human and murine fibroblasts. J Cell Biol **66:** 451-457.

GOSPODAROWICZ, D. B., H; GREENBURG, G, 1978 Purification of the fibroblast growth factor activity from bovine brain. J Biol Chem **253:** 3736-3743.

GRAFF, J. M., 1997 Embryonic patterning: to BMP or not to BMP, that is the question. Cell **89:** 171-174.

GREENMAN, C., P. STEPHENS, R. SMITH, G. L. DALGLIESH, C. HUNTER *et al.*, 2007 Patterns of somatic mutation in human cancer genomes. Nature **446:** 153-158.

GRYZIK, T., and H.-A. J. MÜLLER, 2004a FGF8-like1 and FGF8-like2 encode putative ligands of the FGF receptor Htl and are required for mesoderm migration in the Drosophila gastrula. Curr Biol **14:** 659-667.

GRYZIK, T., and H. A. MÜLLER, 2004b FGF8-like1 and FGF8-like2 encode putative ligands of the FGF receptor Htl and are required for mesoderm migration in the Drosophila gastrula. Curr Biol **14:** 659-667.

GUO, Q., K. LI, N. A. SUNMONU and J. Y. H. LI, 2010 Fgf8b-containing spliceforms, but not Fgf8a, are essential for Fgf8 function during development of the midbrain and cerebellum. Dev Biol **338:** 183-192.

HÄCKER, U., K. NYBAKKEN and N. PERRIMON, 2005 Heparan sulphate proteoglycans: the sweet side of development. Nat Rev Mol Cell Biol **6:** 530-541.

HACOHEN, N., S. KRAMER, D. SUTHERLAND, Y. HIROMI and M. A. KRASNOW, 1998 Sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. Cell **92:** 253-263.

HARADA, M., H. MURAKAMI, A. OKAWA, N. OKIMOTO, S. HIRAOKA *et al.*, 2009 FGF9 monomer-dimer equilibrium regulates extracellular matrix affinity and tissue diffusion. Nat Genet **41:** 289-298.

HARTMANN, T., S. C. BIEGER, B. BRÜHL, P. J. TIENARI, N. IDA *et al.*, 1997 Distinct sites of intracellular production for Alzheimer's disease A beta40/42 amyloid peptides. Nat Med **3:** 1016-1020.

HOFFMAN, R., 1940 Growth **4:** 361-376.

HOLLAND, P. W., J. GARCIA-FERNÀNDEZ, N. A. WILLIAMS and A. SIDOW, 1994 Gene duplications and the origins of vertebrate development. Dev Suppl**:** 125-133.

HOTTA, Y., S. SASAKI, M. KONISHI, H. KINOSHITA, K. KUWAHARA *et al.*, 2008 Fgf16 is required for cardiomyocyte proliferation in the mouse embryonic heart. Dev Dyn **237:** 2947-2954.

HUANG, P., and M. J. STERN, 2004 FGF signaling functions in the hypodermis to regulate fluid balance in C. elegans. Development **131:** 2595-2604.

IKUTA, T., and H. SAIGA, 2007 Dynamic change in the expression of developmental genes in the ascidian central nervous system: revisit to the tripartite model and the origin of the midbrain-hindbrain boundary region. Dev Biol **312:** 631-643.

IMAI, K. S., M. LEVINE, N. SATOH and Y. SATOU, 2006 Regulatory blueprint for a chordate embryo. Science **312:** 1183-1187.

IMAI, K. S., N. SATOH and Y. SATOU, 2002 Early embryonic expression of FGF4/6/9 gene and its role in the induction of mesenchyme and notochord in Ciona savignyi embryos. Development **129:** 1729-1738.

IMAI, K. S., A. STOLFI, M. LEVINE and Y. SATOU, 2009 Gene regulatory networks underlying the compartmentalization of the Ciona central nervous system. Development **136:** 285-293.

IMAM, F., D. SUTHERLAND, W. HUANG and M. A. KRASNOW, 1999 stumps, a Drosophila gene required for fibroblast growth factor (FGF)-directed migrations of tracheal and mesodermal cells. Genetics **152:** 307-318.

ITOH, N., and D. M. ORNITZ, 2008 Functional evolutionary history of the mouse Fgf gene family. Dev Dyn **237:** 18-27.

KADAM, S., A. MCMAHON, P. TZOU and A. STATHOPOULOS, 2009 FGF ligands in Drosophila have distinct activities required to support cell migration and differentiation. Development **136:** 739-747.

KALININA, J., S. A. BYRON, H. P. MAKARENKOVA, S. K. OLSEN, A. V. ELISEENKOVA *et al.*, 2009 Homodimerization controls the fibroblast growth factor 9 subfamily's receptor binding and heparan sulfate-dependent diffusion in the extracellular matrix. Mol Cell Biol **29:** 4663-4678.

KAMIMURA, K., M. FUJISE, F. VILLA, S. IZUMI, H. HABUCHI *et al.*, 2001 Drosophila heparan sulfate 6-O-sulfotransferase (dHS6ST) gene. Structure, expression,

and function in the formation of the tracheal system. J Biol Chem **276:** 17014-17021.

KIRIKOSHI, H., N. SAGARA, T. SAITOH, K. TANAKA, H. SEKIHARA *et al.*, 2000 Molecular cloning and characterization of human FGF-20 on chromosome 8p21.3-p22. Biochem Biophys Res Commun **274:** 337-343.

KLÄMBT, C., L. GLAZER and B. Z. SHILO, 1992 Breathless, a Drosophila FGF receptor homolog, is essential for migration of tracheal and specific midline glial cells. Genes Dev **6:** 1668-1678.

KLINGSEISEN, A., I. B. CLARK, T. GRYZIK and H. A. MÜLLER, 2009a Differential and overlapping functions of two closely related Drosophila FGF8-like growth factors in mesoderm development. Development **136:** 2393-2402.

KLINGSEISEN, A., I. B. N. CLARK, T. GRYZIK and H.-A. J. MÜLLER, 2009b Differential and overlapping functions of two closely related Drosophila FGF8-like growth factors in mesoderm development. Development **136:** 2393-2402.

KOBBERUP, S., M. SCHMERR, M.-L. DANG, P. NYENG, J. N. JENSEN *et al.*, 2010 Conditional control of the differentiation competence of pancreatic endocrine and ductal cells by Fgf10. Mech Dev **127:** 220-234.

KOURAKIS, M. J., and W. C. SMITH, 2007 A conserved role for FGF signaling in chordate otic/atrial placode formation. Dev Biol **312:** 245-257.

KRAMER, S., M. OKABE, N. HACOHEN, M. A. KRASNOW and Y. HIROMI, 1999 Sprouty: a common antagonist of FGF and EGF signaling pathways in Drosophila. Development **126:** 2515-2525.

KÜNNAPUU, J., I. BJÖRKGREN and O. SHIMMI, 2009 The Drosophila DPP signal is produced by cleavage of its proprotein at evolutionary diversified furin-recognition sites. Proc Natl Acad Sci USA **106:** 8501-8506.

KURITA, Y., R. TSUBOI, R. UEKI, D. B. RIFKIN and H. OGAWA, 1992 Immunohistochemical localization of basic fibroblast growth factor in wound healing sites of mouse skin. Arch Dermatol Res **284:** 193-197.

KYTE, J., and R. F. DOOLITTLE, 1982 A simple method for displaying the hydropathic character of a protein. J Mol Biol **157:** 105-132.

LAEZZA, F., A. LAMPERT, M. A. KOZEL, B. R. GERBER, A. M. RUSH *et al.*, 2009 FGF14 N-terminal splice variants differentially modulate Nav1.2 and Nav1.6-encoded sodium channels. Mol Cell Neurosci **42:** 90-101.

LANDER, A. D., and S. B. SELLECK, 2000 The elusive functions of proteoglycans: in vivo veritas. J Cell Biol **148:** 227-232.

LAPRAZ, F., E. RÖTTINGER, V. DUBOC, R. RANGE, L. DULOQUIN *et al.*, 2006 RTK and TGF-beta signaling pathways genes in the sea urchin genome. Dev Biol **300:** 132-152.

LAUTIER, C., S. GOLDWURM, A. DÜRR, B. GIOVANNONE, W. G. TSIARAS *et al.*, 2008 Mutations in the GIGYF2 (TNRC15) gene at the PARK11 locus in familial Parkinson disease. Am J Hum Genet **82:** 822-833.

LAVINE, K. J., and D. M. ORNITZ, 2008 Fibroblast growth factors and Hedgehogs: at the heart of the epicardial signaling center. Trends Genet **24:** 33-40.

LAVINE, K. J., K. YU, A. C. WHITE, X. ZHANG, C. SMITH *et al.*, 2005 Endocardial and epicardial derived FGF signals regulate myocardial proliferation and differentiation in vivo. Dev Cell **8:** 85-95.

LAWRENCE, P. A., R. BODMER and J. P. VINCENT, 1995 Segmental patterning of heart precursors in Drosophila. Development **121:** 4303-4308.

LEE, J. R., S. URBAN, C. F. GARVEY and M. FREEMAN, 2001 Regulated intracellular ligand transport and proteolysis control EGF signal activation in Drosophila. Cell **107:** 161-171.

LEE, P. L., D. E. JOHNSON, L. S. COUSENS, V. A. FRIED and L. T. WILLIAMS, 1989 Purification and complementary DNA cloning of a receptor for basic fibroblast growth factor. Science **245:** 57-60.

LEMMON, S. K., M. C. RILEY, K. A. THOMAS, G. A. HOOVER, T. MACIAG *et al.*, 1982 Bovine fibroblast growth factor: comparison of brain and pituitary preparations. J Cell Biol **95:** 162-169.

LEMOSY, E. K., 2006 Proteolytic regulatory mechanisms in the formation of extracellular morphogen gradients. Birth Defects Res C Embryo Today **78:** 243-255.

LIBERMAN, L. M., and A. STATHOPOULOS, 2009 Design flexibility in cis-regulatory control of gene expression: synthetic and comparative evidence. Dev Biol **327:** 578-589.

LIBERMANN, T. A., R. FRIESEL, M. JAYE, R. M. LYALL, B. WESTERMARK *et al.*, 1987 An angiogenic growth factor is expressed in human glioma cells. EMBO J **6:** 1627-1632.

LIEBER, T., S. KIDD and M. W. YOUNG, 2002 Kuzbanian-mediated cleavage of Drosophila Notch. Genes Dev **16:** 209-221.

LIN, X., E. M. BUFF, N. PERRIMON and A. M. MICHELSON, 1999 Heparan sulfate proteoglycans are essential for FGF receptor signaling during Drosophila embryonic development. Development **126:** 3715-3723.

LIN, X., and N. PERRIMON, 2000 Role of heparan sulfate proteoglycans in cell-cell signaling in Drosophila. Matrix Biol **19:** 303-307.

LIN, X., and N. PERRIMON, 2002 Developmental roles of heparan sulfate proteoglycans in Drosophila. Glycoconj J **19:** 363-368.

LIU, A., J. Y. H. LI, C. BROMLEIGH, Z. LAO, L. A. NISWANDER *et al.*, 2003 FGF17b and FGF18 have different midbrain regulatory properties from FGF8b or activated FGF receptors. Development **130:** 6175-6185.

LIU, A., K. LOSOS and A. L. JOYNER, 1999 FGF8 can activate Gbx2 and transform regions of the rostral mouse brain into a hindbrain fate. Development **126:** 4827-4838.

LO, T.-W., C. S. BRANDA, P. HUANG, I. E. SASSON, S. J. GOODMAN *et al.*, 2008 Different isoforms of the C. elegans FGF receptor are required for attraction and repulsion of the migrating sex myoblasts. Dev Biol **318:** 268-275.

LOGEAT, F., C. BESSIA, C. BROU, O. LEBAIL, S. JARRIAULT *et al.*, 1998 The Notch1 receptor is cleaved constitutively by a furin-like convertase. Proc Natl Acad Sci USA **95:** 8108-8112.

LU, W., Y. LUO, M. KAN and W. L. MCKEEHAN, 1999 Fibroblast growth factor-10. A second candidate stromal to epithelial cell andromedin in prostate. J Biol Chem **274:** 12827-12834.

MACIAG, T., J. CERUNDOLO, S. ILSLEY, P. R. KELLEY and R. FORAND, 1979 An endothelial cell growth factor from bovine hypothalamus: identification and partial characterization. Proc Natl Acad Sci USA **76:** 5674-5678.

MANDAL, L., K. DUMSTREI and V. HARTENSTEIN, 2004 Role of FGFR signaling in the morphogenesis of the Drosophila visceral musculature. Dev Dyn **231:** 342-348.

MARCHESE, C., M. CHEDID, O. R. DIRSCH, K. G. CSAKY, F. SANTANELLI *et al.*, 1995 Modulation of keratinocyte growth factor and its receptor in reepithelializing human skin. J Exp Med **182:** 1369-1376.

MARICS, I., J. ADELAIDE, F. RAYBAUD, M. G. MATTEI, F. COULIER *et al.*, 1989 Characterization of the HST-related FGF.6 gene, a new member of the fibroblast growth factor gene family. Oncogene **4:** 335-340.

MARIE, P. J., 2003 Fibroblast growth factor signaling controlling osteoblast differentiation. Gene **316:** 23-32.

MARTINEZ, S., P. H. CROSSLEY, I. COBOS, J. L. RUBENSTEIN and G. R. MARTIN, 1999 FGF8 induces formation of an ectopic isthmic organizer and isthmocerebellar development via a repressive effect on Otx2 expression. Development **126:** 1189-1200.

MARUOKA, Y., N. OHBAYASHI, M. HOSHIKAWA, N. ITOH, B. L. HOGAN *et al.*, 1998 Comparison of the expression of three highly related genes, Fgf8, Fgf17 and Fgf18, in the mouse embryo. Mech Dev **74:** 175-177.

MATUS, D. Q., G. H. THOMSEN and M. Q. MARTINDALE, 2007 FGF signaling in gastrulation and neural development in Nematostella vectensis, an anthozoan cnidarian. Dev Genes Evol **217:** 137-148.

MCCOON, P. E., R. C. ANGERER and L. M. ANGERER, 1996 SpFGFR, a new member of the fibroblast growth factor receptor family, is developmentally regulated during early sea urchin development. J Biol Chem **271:** 20119-20125.

MCCOON, P. E., E. BLACKSTONE, R. C. ANGERER and L. M. ANGERER, 1998 Sea urchin FGFR muscle-specific expression: posttranscriptional regulation in embryos and adults. Dev Biol **200:** 171-181.

MCMAHON, A., W. SUPATTO, S. E. FRASER and A. STATHOPOULOS, 2008 Dynamic analyses of Drosophila gastrulation provide insights into collective cell migration. Science **322:** 1546-1550.

MEDINA, M., A. G. COLLINS, J. D. SILBERMAN and M. L. SOGIN, 2001 Evaluating hypotheses of basal animal phylogeny using complete sequences of large and small subunit rRNA. Proc Natl Acad Sci USA **98:** 9707-9712.

MEYERS, E. N., M. LEWANDOSKI and G. R. MARTIN, 1998 An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. Nat Genet **18:** 136-141.

MICHELSON, A. M., S. GISSELBRECHT, E. BUFF and J. B. SKEATH, 1998a Heartbroken is a specific downstream mediator of FGF receptor signalling in Drosophila. Development **125:** 4379-4389.

MICHELSON, A. M., S. GISSELBRECHT, Y. ZHOU, K. H. BAEK and E. M. BUFF, 1998b Dual functions of the heartless fibroblast growth factor receptor in development of the Drosophila embryonic mesoderm. Dev Genet **22:** 212-229.

MIKI, T., D. P. BOTTARO, T. P. FLEMING, C. L. SMITH, W. H. BURGESS *et al.*, 1992 Determination of ligand-binding specificity by alternative splicing: two distinct growth factor receptors encoded by a single gene. Proc Natl Acad Sci USA **89:** 246-250.

MINOWADA, G., L. A. JARVIS, C. L. CHI, A. NEUBÜSER, X. SUN *et al.*, 1999 Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. Development **126:** 4465-4475.

MIYAKE, A., M. KONISHI, F. H. MARTIN, N. A. HERNDAY, K. OZAKI *et al.*, 1998 Structure and expression of a novel member, FGF-16, on the fibroblast growth factor family. Biochem Biophys Res Commun **243:** 148-152.

MIYAMOTO, M., K. NARUO, C. SEKO, S. MATSUMOTO, T. KONDO *et al.*, 1993 Molecular cloning of a novel cytokine cDNA encoding the ninth member of the fibroblast growth factor family, which has a unique secretion property. Mol Cell Biol **13:** 4251-4259.

MOHAMMADI, M., I. DIKIC, A. SOROKIN, W. H. BURGESS, M. JAYE *et al.*, 1996 Identification of six novel autophosphorylation sites on fibroblast growth factor receptor 1 and elucidation of their importance in receptor activation and signal transduction. Mol Cell Biol **16:** 977-989.

MOHAMMADI, M., S. K. OLSEN and O. A. IBRAHIMI, 2005 Structural basis for fibroblast growth factor receptor activation. Cytokine Growth Factor Rev **16:** 107-137.

MOLLOY, S. S., P. A. BRESNAHAN, S. H. LEPPLA, K. R. KLIMPEL and G. THOMAS, 1992 Human furin is a calcium-dependent serine endoprotease that recognizes the sequence Arg-X-X-Arg and efficiently cleaves anthrax toxin protective antigen. J Biol Chem **267:** 16396-16402.

MONTERO, A., Y. OKADA, M. TOMITA, M. ITO, H. TSURUKAMI *et al.*, 2000 Disruption of the fibroblast growth factor-2 gene results in decreased bone mass and bone formation. J Clin Invest **105:** 1085-1093.

MORISATO, D., 2001 Spätzle regulates the shape of the Dorsal gradient in the Drosophila embryo. Development **128:** 2309-2319.

MORISATO, D., and K. V. ANDERSON, 1994 The spätzle gene encodes a component of the extracellular signaling pathway establishing the dorsal-ventral pattern of the Drosophila embryo. Cell **76:** 677-688.

MUMM, J. S., E. H. SCHROETER, M. T. SAXENA, A. GRIESEMER, X. TIAN *et al.*, 2000 A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1. Mol Cell **5:** 197-206.

MURAKAMI, M., L. T. NGUYEN, Z. W. ZHUANG, Z. W. ZHANG, K. L. MOODIE *et al.*, 2008 The FGF system has a key role in regulating vascular integrity. J Clin Invest **118:** 3355-3366.

NAKATAKE, Y., M. HOSHIKAWA, T. ASAKI, Y. KASSAI and N. ITOH, 2001 Identification of a novel fibroblast growth factor, FGF-22, preferentially expressed in the inner root sheath of the hair follicle. Biochim Biophys Acta **1517:** 460-463.

NEUMANN, C., and S. COHEN, 1997 Morphogens and pattern formation. Bioessays **19:** 721-729.

NISHIMURA, T., Y. UTSUNOMIYA, M. HOSHIKAWA, H. OHUCHI and N. ITOH, 1999 Structure and expression of a novel human FGF, FGF-19, expressed in the fetal brain. Biochim Biophys Acta **1444:** 148-151.

NISWANDER, L., C. TICKLE, A. VOGEL, I. BOOTH and G. R. MARTIN, 1993 FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. Cell **75:** 579-587.

OGAWA, K., C. KOBAYASHI, T. HAYASHI, H. ORII, K. WATANABE *et al.*, 2002 Planarian fibroblast growth factor receptor homologs expressed in stem cells and cephalic ganglions. Dev Growth Differ **44:** 191-204.

OHBAYASHI, N., M. HOSHIKAWA, S. KIMURA, M. YAMASAKI, S. FUKUI *et al.*, 1998 Structure and expression of the mRNA encoding a novel fibroblast growth factor, FGF-18. J Biol Chem **273:** 18161-18164.

OHNO, S., 1970 *Evolution by Gene Duplication*. Springer-Verlag, Berlin-Heidelberg-New York.

OLSEN, S. K., M. GARBI, N. ZAMPIERI, A. V. ELISEENKOVA, D. M. ORNITZ *et al.*, 2003 Fibroblast growth factor (FGF) homologous factors share structural but not functional homology with FGFs. J Biol Chem **278:** 34226-34236.

OLWIN, B. B., and S. D. HAUSCHKA, 1986 Identification of the fibroblast growth factor receptor of Swiss 3T3 cells and mouse skeletal muscle myoblasts. Biochemistry **25:** 3487-3492.

ORNITZ, D. M., and N. ITOH, 2001a Fibroblast growth factors. Genome Biol **2:** REVIEWS3005.

ORNITZ, D. M., and N. ITOH, 2001b Fibroblast growth factors. Genome Biol **2:** reviews3005.3001-3005.3012.

ORNITZ, D. M., J. XU, J. S. COLVIN, D. G. MCEWEN, C. A. MACARTHUR *et al.*, 1996 Receptor specificity of the fibroblast growth factor family. J Biol Chem **271:** 15292-15297.

ORR-URTREGER, A., M. T. BEDFORD, T. BURAKOVA, E. ARMAN, Y. ZIMMER *et al.*, 1993 Developmental localization of the splicing alternatives of fibroblast growth factor receptor-2 (FGFR2). Dev Biol **158:** 475-486.

ORTEGA, S., M. ITTMANN, S. H. TSANG, M. EHRLICH and C. BASILICO, 1998 Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. Proc Natl Acad Sci USA **95:** 5672-5677.

PANGANIBAN, G. E., K. E. RASHKA, M. D. NEITZEL and F. M. HOFFMANN, 1990 Biochemical characterization of the Drosophila dpp protein, a member of the transforming growth factor beta family of growth factors. Mol Cell Biol **10:** 2669-2677.

PARK, M., X. WU, K. GOLDEN, J. D. AXELROD and R. BODMER, 1996 The wingless signaling pathway is directly involved in Drosophila heart development. Dev Biol **177:** 104-116.

PELLEGRINI, L., D. F. BURKE, F. VON DELFT, B. MULLOY and T. L. BLUNDELL, 2000 Crystal structure of fibroblast growth factor receptor ectodomain bound to ligand and heparin. Nature **407:** 1029-1034.

PERRIMON, N., and M. BERNFIELD, 2000 Specificities of heparan sulphate proteoglycans in developmental processes. Nature **404:** 725-728.

PLOTNIKOV, A. N., A. V. ELISEENKOVA, O. A. IBRAHIMI, Z. SHRIVER, R. SASISEKHARAN *et al.*, 2001 Crystal structure of fibroblast growth factor 9 reveals regions implicated in dimerization and autoinhibition. J Biol Chem **276:** 4322-4329.

POLANSKA, U. M., D. G. FERNIG and T. KINNUNEN, 2009 Extracellular interactome of the FGF receptor-ligand system: complexities and the relative simplicity of the worm. Dev Dyn **238:** 277-293.

POPOVICI, C., F. CONCHONAUD, D. BIRNBAUM and R. ROUBIN, 2004 Functional phylogeny relates LET-756 to fibroblast growth factor 9. J Biol Chem **279:** 40146-40152.

POPOVICI, C., M. FALLET, D. MARGUET, D. BIRNBAUM and R. ROUBIN, 2006 Intracellular trafficking of LET-756, a fibroblast growth factor of C. elegans, is controlled by a balance of export and nuclear signals. Exp Cell Res **312:** 1484-1495.

POPOVICI, C., R. ROUBIN, F. COULIER and D. BIRNBAUM, 2005 An evolutionary history of the FGF superfamily. Bioessays **27:** 849-857.

PRESTA, M., P. DELL'ERA, S. MITOLA, E. MORONI, R. RONCA *et al.*, 2005 Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. Cytokine Growth Factor Rev **16:** 159-178.

PUTNAM, N. H., M. SRIVASTAVA, U. HELLSTEN, B. DIRKS, J. CHAPMAN *et al.*, 2007 Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization. Science **317:** 86-94.

RAAIJMAKERS, J. H., and J. L. BOS, 2009 Specificity in Ras and Rap signaling. J Biol Chem **284:** 10995-10999.

RAND, M. D., L. M. GRIMM, S. ARTAVANIS-TSAKONAS, V. PATRIUB, S. C. BLACKLOW *et al.*, 2000 Calcium depletion dissociates and activates heterodimeric notch receptors. Mol Cell Biol **20:** 1825-1835.

RAPRAEGER, A. C., A. KRUFKA and B. B. OLWIN, 1991 Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. Science **252:** 1705-1708.

REIFERS, F., H. BÖHLI, E. C. WALSH, P. H. CROSSLEY, D. Y. STAINIER *et al.*, 1998 Fgf8 is mutated in zebrafish acerebellar (ace) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. Development **125:** 2381-2395.

RENTZSCH, F., J. H. FRITZENWANKER, C. B. SCHOLZ and U. TECHNAU, 2008 FGF signalling controls formation of the apical sensory organ in the cnidarian Nematostella vectensis. Development **135:** 1761-1769.

RHOLAM, M., and C. FAHY, 2009 Processing of peptide and hormone precursors at the dibasic cleavage sites. Cell Mol Life Sci **66:** 2075-2091.

RÖTTINGER, E., A. SAUDEMONT, V. DUBOC, L. BESNARDEAU, D. MCCLAY *et al.*, 2008 FGF signals guide migration of mesenchymal cells, control skeletal morphogenesis [corrected] and regulate gastrulation during sea urchin development. Development **135:** 353-365.

ROUBIN, R., K. NAERT, C. POPOVICI, G. VATCHER, F. COULIER *et al.*, 1999 let-756, a C. elegans fgf essential for worm development. Oncogene **18:** 6741-6747.

ROUSSEAU, F., J. BONAVENTURE, L. LEGEAI-MALLET, A. PELET, J. M. ROZET *et al.*, 1994 Mutations in the gene encoding fibroblast growth factor receptor-3 in achondroplasia. Nature **371:** 252-254.

RUBIN, J. S., H. OSADA, P. W. FINCH, W. G. TAYLOR, S. RUDIKOFF *et al.*, 1989 Purification and characterization of a newly identified growth factor specific for epithelial cells. Proc Natl Acad Sci USA **86:** 802-806.

RUTLEDGE, B. J., K. ZHANG, E. BIER, Y. N. JAN and N. PERRIMON, 1992 The Drosophila spitz gene encodes a putative EGF-like growth factor involved in dorsal-ventral axis formation and neurogenesis. Genes Dev **6:** 1503-1517.

RYAN, J. F., P. M. BURTON, M. E. MAZZA, G. K. KWONG, J. C. MULLIKIN *et al.*, 2006 The cnidarian-bilaterian ancestor possessed at least 56 homeoboxes: evidence from the starlet sea anemone, Nematostella vectensis. Genome Biol **7:** R64.

SAKAMOTO, H., M. MORI, M. TAIRA, T. YOSHIDA, S. MATSUKAWA *et al.*, 1986 Transforming gene from human stomach cancers and a noncancerous portion of stomach mucosa. Proc Natl Acad Sci USA **83:** 3997-4001.

SALVESEN, G. S., AND NAGASE, H., 2001 Inhibition of Proteolytic Enzymes in *Proteokytic Enzymes*, edited by R. A. B. J. S. BEYNON. Oxford University Press, Oxford.

SATO, T., I. ARAKI and H. NAKAMURA, 2001 Inductive signal and tissue responsiveness defining the tectum and the cerebellum. Development **128:** 2461-2469.

SATOU, Y., K. S. IMAI and N. SATOH, 2002 Fgf genes in the basal chordate Ciona intestinalis. Dev Genes Evol **212:** 432-438.

SAUNDERS, J. W., 1948 The proximo-distal sequence of origin of the parts of the chick wing and the role of the ectoderm. J Exp Zool **108:** 363-403.

SAWADA, A., M. SHINYA, Y. J. JIANG, A. KAWAKAMI, A. KUROIWA *et al.*, 2001 Fgf/MAPK signalling is a crucial positional cue in somite boundary formation. Development **128:** 4873-4880.

SCHMID, K. J., and D. TAUTZ, 1999 A comparison of homologous developmental genes from Drosophila and Tribolium reveals major differences in length and trinucleotide repeat content. J Mol Evol **49:** 558-566.

SCHOLPP, S., and M. BRAND, 2004 Endocytosis controls spreading and effective signaling range of Fgf8 protein. Curr Biol **14:** 1834-1841.

SCHWEITZER, R., M. SHAHARABANY, R. SEGER and B. Z. SHILO, 1995 Secreted Spitz triggers the DER signaling pathway and is a limiting component in embryonic ventral ectoderm determination. Genes Dev **9:** 1518-1529.

SEIDAH, N. G., and M. CHRÉTIEN, 1999 Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. Brain Res **848:** 45-62.

SHAMIM, H., R. MAHMOOD, C. LOGAN, P. DOHERTY, A. LUMSDEN *et al.*, 1999 Sequential roles for Fgf4, En1 and Fgf8 in specification and regionalisation of the midbrain. Development **126:** 945-959.

SHEIKH, H., and I. MASON, 1996 Polarising activity of FGF-8 in the avian midbrain. Int J Dev Biol **Suppl 1:** 117S-118S.

SHI, W., S. M. PEYROT, E. MUNRO and M. LEVINE, 2009 FGF3 in the floor plate directs notochord convergent extension in the Ciona tadpole. Development **136:** 23-28.

SHIANG, R., L. M. THOMPSON, Y. Z. ZHU, D. M. CHURCH, T. J. FIELDER *et al.*, 1994 Mutations in the transmembrane domain of FGFR3 cause the most common genetic form of dwarfism, achondroplasia. Cell **78:** 335-342.

SHIMMI, O., D. UMULIS, H. OTHMER and M. B. O'CONNOR, 2005 Facilitated transport of a Dpp/Scw heterodimer by Sog/Tsg leads to robust patterning of the Drosophila blastoderm embryo. Cell **120:** 873-886.

SHISHIDO, E., N. ONO, T. KOJIMA and K. SAIGO, 1997 Requirements of DFR1/Heartless, a mesoderm-specific Drosophila FGF-receptor, for the

formation of heart, visceral and somatic muscles, and ensheathing of longitudinal axon tracts in CNS. Development **124:** 2119-2128.

SIVAK, J. M., L. F. PETERSEN and E. AMAYA, 2005 FGF signal interpretation is directed by Sprouty and Spred proteins during mesoderm formation. Dev Cell **8:** 689-701.

SLACK, J. M., B. G. DARLINGTON, J. K. HEATH and S. F. GODSAVE, 1987 Mesoderm induction in early Xenopus embryos by heparin-binding growth factors. Nature **326:** 197-200.

SLEEMAN, M., J. FRASER, M. MCDONALD, S. YUAN, D. WHITE *et al.*, 2001 Identification of a new fibroblast growth factor receptor, FGFR5. Gene **271:** 171-182.

SMALLWOOD, P. M., I. MUNOZ-SANJUAN, P. TONG, J. P. MACKE, S. H. HENDRY *et al.*, 1996 Fibroblast growth factor (FGF) homologous factors: new members of the FGF family implicated in nervous system development. Proc Natl Acad Sci USA **93:** 9850-9857.

STAHL, M., R. SCHUH and B. ADRYAN, 2007 Identification of FGF-dependent genes in the Drosophila tracheal system. Gene Expr Patterns **7:** 202-209.

STATHOPOULOS, A., B. TAM, M. RONSHAUGEN, M. FRASCH and M. LEVINE, 2004 Pyramus and thisbe: FGF genes that pattern the mesoderm of Drosophila embryos. Genes Dev **18:** 687-699.

STATHOPOULOS, A., M. VAN DRENTH, A. ERIVES, M. MARKSTEIN and M. LEVINE, 2002 Whole-genome analysis of dorsal-ventral patterning in the Drosophila embryo. Cell **111:** 687-701.

STEINBERG, F., L. ZHUANG, M. BEYELER, R. E. KÄLIN, P. E. MULLIS *et al.*, 2010 The FGFRL1 receptor is shed from cell membranes, binds fibroblast growth factors (FGFs), and antagonizes FGF signaling in Xenopus embryos. J Biol Chem **285:** 2193-2202.

STERN, M. J., and H. R. HORVITZ, 1991 A normally attractive cell interaction is repulsive in two C. elegans mesodermal cell migration mutants. Development **113:** 797-803.

STIENEKE-GRÖBER, A., M. VEY, H. ANGLIKER, E. SHAW, G. THOMAS *et al.*, 1992 Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBO J **11:** 2407-2414.

SUDHOP, S., F. COULIER, A. BIELLER, A. VOGT, T. HOTZ *et al.*, 2004 Signalling by the FGFR-like tyrosine kinase, Kringelchen, is essential for bud detachment in Hydra vulgaris. Development **131:** 4001-4011.

SUMMERBELL, D., 1974 A quantitative analysis of the effect of excision of the AER from the chick limb-bud. J Embryol Exp Morphol **32:** 651-660.

SUN, X., E. N. MEYERS, M. LEWANDOSKI and G. R. MARTIN, 1999 Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. Genes Dev **13:** 1834-1846.

SUTHERLAND, D., C. SAMAKOVLIS and M. A. KRASNOW, 1996 branchless encodes a Drosophila FGF homolog that controls tracheal cell migration and the pattern of branching. Cell **87:** 1091-1101.

TANAKA, A., K. MIYAMOTO, N. MINAMINO, M. TAKEDA, B. SATO *et al.*, 1992 Cloning and characterization of an androgen-induced growth factor essential for the

androgen-dependent growth of mouse mammary carcinoma cells. Proc Natl Acad Sci USA **89:** 8928-8932.

TANIGUCHI, K., K.-I. SASAKI, K. WATARI, H. YASUKAWA, T. IMAIZUMI *et al.*, 2009 Suppression of Sproutys has a therapeutic effect for a mouse model of ischemia by enhancing angiogenesis. PLoS ONE **4:** e5467.

TECHNAU, U., S. RUDD, P. MAXWELL, P. M. K. GORDON, M. SAINA *et al.*, 2005 Maintenance of ancestral complexity and non-metazoan genes in two basal cnidarians. Trends Genet **21:** 633-639.

THISSE, B., and C. THISSE, 2005 Functions and regulations of fibroblast growth factor signaling during embryonic development. Dev Biol **287:** 390-402.

TROWELL OA, C. B., WILLMER EN, 1939 J Exp Biol **16:** 60-70.

TULIN, S., and A. SATHOPOULOS, 2010b Extending the family table: insights from beyond vertebrates into the regulation of embryonic development by FGFs. Birth Defects Research Part C: Embryo Today **90:** 214-227.

TULIN, S., and A. STATHOPOULOS, 2010a Analysis of Thisbe and Pyramus functional domains reveals evidence for cleavage of Drosophila FGFs. BMC Dev Biol **10:** 83.

TURNER, N., and R. GROSE, 2010 Fibroblast growth factor signalling: from development to cancer. Nat Rev Cancer **10:** 116-129.

URAKAWA, I., Y. YAMAZAKI, T. SHIMADA, K. IIJIMA, H. HASEGAWA *et al.*, 2006 Klotho converts canonical FGF receptor into a specific receptor for FGF23. Nature **444:** 770-774.

URBACH, R., 2007 A procephalic territory in Drosophila exhibiting similarities and dissimilarities compared to the vertebrate midbrain/hindbrain boundary region. Neural development **2:** 23.

URBAN, S., J. R. LEE and M. FREEMAN, 2001 Drosophila rhomboid-1 defines a family of putative intramembrane serine proteases. Cell **107:** 173-182.

VAN DEN STEEN, P., P. M. RUDD, R. A. DWEK and G. OPDENAKKER, 1998 Concepts and principles of O-linked glycosylation. Crit Rev Biochem Mol Biol **33:** 151-208.

VINCENT, S., R. WILSON, C. COELHO, M. AFFOLTER and M. LEPTIN, 1998 The Drosophila protein Dof is specifically required for FGF signaling. Mol Cell **2:** 515-525.

WANG, K. K., R. NATH, A. POSNER, K. J. RASER, M. BUROKER-KILGORE *et al.*, 1996 An alpha-mercaptoacrylic acid derivative is a selective nonpeptide cell-permeable calpain inhibitor and is neuroprotective. Proc Natl Acad Sci USA **93:** 6687-6692.

WEBER, A. N., S. TAUSZIG-DELAMASURE, J. A. HOFFMANN, E. LELIÈVRE, H. GASCAN *et al.*, 2003 Binding of the Drosophila cytokine Spätzle to Toll is direct and establishes signaling. Nat Immunol **4:** 794-800.

WEINSTEIN, M., X. XU, K. OHYAMA and C. X. DENG, 1998 FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung. Development **125:** 3615-3623.

WERNER, S., K. G. PETERS, M. T. LONGAKER, F. FULLER-PACE, M. J. BANDA *et al.*, 1992 Large induction of keratinocyte growth factor expression in the dermis during wound healing. Proc Natl Acad Sci USA **89:** 6896-6900.

WHARTON, K., and R. DERYNCK, 2009 TGFbeta family signaling: novel insights in development and disease. Development **136:** 3691-3697.

WHITELOCK, J. M., and R. V. IOZZO, 2005 Heparan sulfate: a complex polymer charged with biological activity. Chem Rev **105:** 2745-2764.

WIEDEMANN, M., and B. TRUEB, 2000 Characterization of a novel protein (FGFRL1) from human cartilage related to FGF receptors. Genomics **69:** 275-279.

WILKIE, A. O. M., 2005 Bad bones, absent smell, selfish testes: the pleiotropic consequences of human FGF receptor mutations. Cytokine Growth Factor Rev **16:** 187-203.

WILLS, A. A., A. R. KIDD, A. LEPILINA and K. D. POSS, 2008 Fgfs control homeostatic regeneration in adult zebrafish fins. Development **135:** 3063-3070.

WILSON, R., E. VOGELSANG and M. LEPTIN, 2005 FGF signalling and the mechanism of mesoderm spreading in Drosophila embryos. Development **132:** 491-501.

WU, X., K. GOLDEN and R. BODMER, 1995 Heart development in Drosophila requires the segment polarity gene wingless. Dev Biol **169:** 619-628.

XU, J., A. LAWSHE, C. A. MACARTHUR and D. M. ORNITZ, 1999 Genomic structure, mapping, activity and expression of fibroblast growth factor 17. Mech Dev **83:** 165-178.

XU, J., Z. LIU and D. M. ORNITZ, 2000 Temporal and spatial gradients of Fgf8 and Fgf17 regulate proliferation and differentiation of midline cerebellar structures. Development **127:** 1833-1843.

XU, X., M. WEINSTEIN, C. LI, M. NASKI, R. I. COHEN *et al.*, 1998 Fibroblast growth factor receptor 2 (FGFR2)-mediated reciprocal regulation loop between FGF8 and FGF10 is essential for limb induction. Development **125:** 753-765.

YAMAGUCHI, T. P., K. HARPAL, M. HENKEMEYER and J. ROSSANT, 1994 fgfr-1 is required for embryonic growth and mesodermal patterning during mouse gastrulation. Genes Dev **8:** 3032-3044.

YAMASHITA, T., M. YOSHIOKA and N. ITOH, 2000 Identification of a novel fibroblast growth factor, FGF-23, preferentially expressed in the ventrolateral thalamic nucleus of the brain. Biochem Biophys Res Commun **277:** 494-498.

YAN, D., and X. LIN, 2007 Drosophila glypican Dally-like acts in FGF-receiving cells to modulate FGF signaling during tracheal morphogenesis. Dev Biol **312:** 203-216.

YASUO, H., and C. HUDSON, 2007 FGF8/17/18 functions together with FGF9/16/20 during formation of the notochord in Ciona embryos. Dev Biol **302:** 92-103.

YAYON, A., Y. ZIMMER, G. H. SHEN, A. AVIVI, Y. YARDEN *et al.*, 1992 A confined variable region confers ligand specificity on fibroblast growth factor receptors: implications for the origin of the immunoglobulin fold. EMBO J **11:** 1885-1890.

YU, C., F. WANG, M. KAN, C. JIN, R. B. JONES *et al.*, 2000 Elevated cholesterol metabolism and bile acid synthesis in mice lacking membrane tyrosine kinase receptor FGFR4. J Biol Chem **275:** 15482-15489.

YU, S., M. BURKHARDT, M. NOWAK, J. RIES, Z. PETRÁŠEK *et al.*, 2009a Fgf8 morphogen gradient forms by a source-sink mechanism with freely diffusing molecules. Nature.

ZHAN, X., B. BATES, X. G. HU and M. GOLDFARB, 1988 The human FGF-5 oncogene encodes a novel protein related to fibroblast growth factors. Mol Cell Biol **8:** 3487-3495.

ZHANG, X., O. A. IBRAHIMI, S. K. OLSEN, H. UMEMORI, M. MOHAMMADI *et al.*, 2006 Receptor specificity of the fibroblast growth factor family. The complete mammalian FGF family. J Biol Chem **281:** 15694-15700.