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Xenopus laevis FGF16 activates the expression of genes coding for the transcription factors Sp5 and Sp5l

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ABSTRACT Fibroblast growth factors (FGFs) comprise a family of signalling molecules with essential roles in early embryonic development across animal species. The role of FGFs in mesoderm formation and patterning in *Xenopus* has been particularly well studied. However, little is known about FGF16 in *Xenopus*. Using *in situ* hybridisation, we uncover the expression pattern of *FGF16* during early *Xenopus laevis* development, which has not been previously described. We show that the zygotic expression of *FGF16* is activated in the mesoderm of the early gastrula as a ring around the blastopore, with its first accumulation at the dorsal side of the embryo. Later, *FGF16* expression is found in the otic vesicle, the branchial arches and the anterior pituitary, as well as in the chordal neural hinge region of the tailbud. In addition, we show that FGF16 can activate the MAPK pathway and expression of *sp5* and *sp5l*. Like *FGF16*, *sp5* is expressed in the otic vesicle and the branchial arches, with all three of these genes being expressed in the tailbud. These data provide evidence that FGF16 is present in the early mesoderm and can activate the expression of developmentally important transcription factors.

KEY WORDS: *mesoderm, secreted growth factor, cell signaling, transcriptional regulation*

Fibroblast growth factors (FGFs) are small polypeptide signalling molecules defined by a conserved 120-140 amino acid core and their high affinity for heparan sulfate (Goetz & Mohammadi, 2013). FGFs signal through a family of tyrosine kinase receptors (Zhang *et al.*, 2006) coded for by four distinct FGF receptor (FGFR) genes that can be differentially spliced (Johnson *et al.*, 1991). This, together with multiple distinct ligands, leads to the impressive complexity underlying FGF signalling. Activation of the FGF pathway involves the formation of a tripartite signalling complex of FGF ligand, heparan sulfate, and FGFR (Turnbull *et al.*, 2001). Receptor dimerization activates one of four downstream signal transduction pathways: mitogen-activated protein kinase (MAPK), phospholipase-C γ1 (PLC-γ1), phosphatidylinositol 3-kinase (PI-3 kinase) or Janus kinase/signal transducer and activator of transcription (Jak/STAT) (Dorey & Amaya, 2010; Ornitz & Itoh, 2015). The autophosphorylation of tyrosine residues within the FGFR intracellular domain results in the phosphorylation of FRS2α, a kinase substrate constitutively associated with the FGFR, and the subsequent recruitment of the GRB2 adapter protein. GRB2 then recruits the guanine nucleotide exchange factor, son of sevenless (SOS) (Ong *et al.*, 2000). For the activation of the MAPK pathway, SOS activates Ras GTPase, in turn activating Raf (a MAP kinase kinase kinase), Mek (a MAP kinase kinase) and MAPK ERK in a cascade of phosphorylation events. Phosphorylated ERK translocates to the nucleus and modifies the activity of Ets family transcription factors, resulting in effects on gene expression downstream of FGF signalling (Randi *et al.*, 2009).

There are 22 members of the FGF family, which are divided into subfamilies: paracrine FGFs (FGF1-10, 16-18, 20, 22), intracellular FGFs (FGF11-14), and endocrine FGFs (FGF15/19, 21,23). Most FGFs require N-terminal signal peptides for secretion (Käll *et al.*, 2004). However, the FGF9 group, including FGF 9, 16 and 20, lack cleavable signal peptides (Itoh & Ornitz, 2004). The secretion of these FGFs relies upon un-cleavable bipartite signals in the N-terminal and central hydrophobic region (Miyakawa *et al.*, 1999; Revest *et al.*, 2000), with secretion, nonetheless, requiring the Golgi and the endoplasmic reticulum (ER) (Miyakawa & Imamura,2003).

FGF16 was originally identified in the rat heart (Miyake *et al.*, 1998) and has known cardiogenic and cardioprotective roles (Hotta *et al.*, 2008; Lu *et al.*, 2008; Wang *et al.*, 2015), as well as roles in the development of the chick inner ear (Chapman *et al.*,

Abbreviations used in this paper: FGF, fibroblast growth factor; MAPK: mitogen-activated protein kinase (also called ERK); Sp5 and Sp5l, proteins related to the human transcription factor Sp1; X.laevis, Xenopus laevis; X.tropicalis, Xenopus tropicalis.

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2006; Olaya-Sánchez *et al.*, 2017), the formation of pectoral fin buds in zebrafish (Nomura *et al.*, 2006), and the specification of GABAergic neurons and oligodendrocytes in the zebrafish forebrain (Miyake *et al.*, 2014). RNA-seq analysis shows *FGF16* is expressed during gastrulation in *X.laevis* and *X.tropicalis* embryos (Owens *et al.*, 2016; Session *et al.*, 2016). For example, *FGF16.L* expression peaks at NF stage 12 and remains high during neurulation in *X.laevis*, before decreasing in early and late tailbud stages, whereas *FGF16.S* is expressed at a much lower level, and is not expressed after gastrulation (Owens *et al.*, 2016; Session *et al.*, 2016). Attempts to clone *FGF16* from *Xenopus tropicalis* were unsuccessful (Lea *et al.*, 2009), and the spatial expression pattern of *FGF16* has not been previously described during the development of *X.tropicalis* or *X.laevis.*

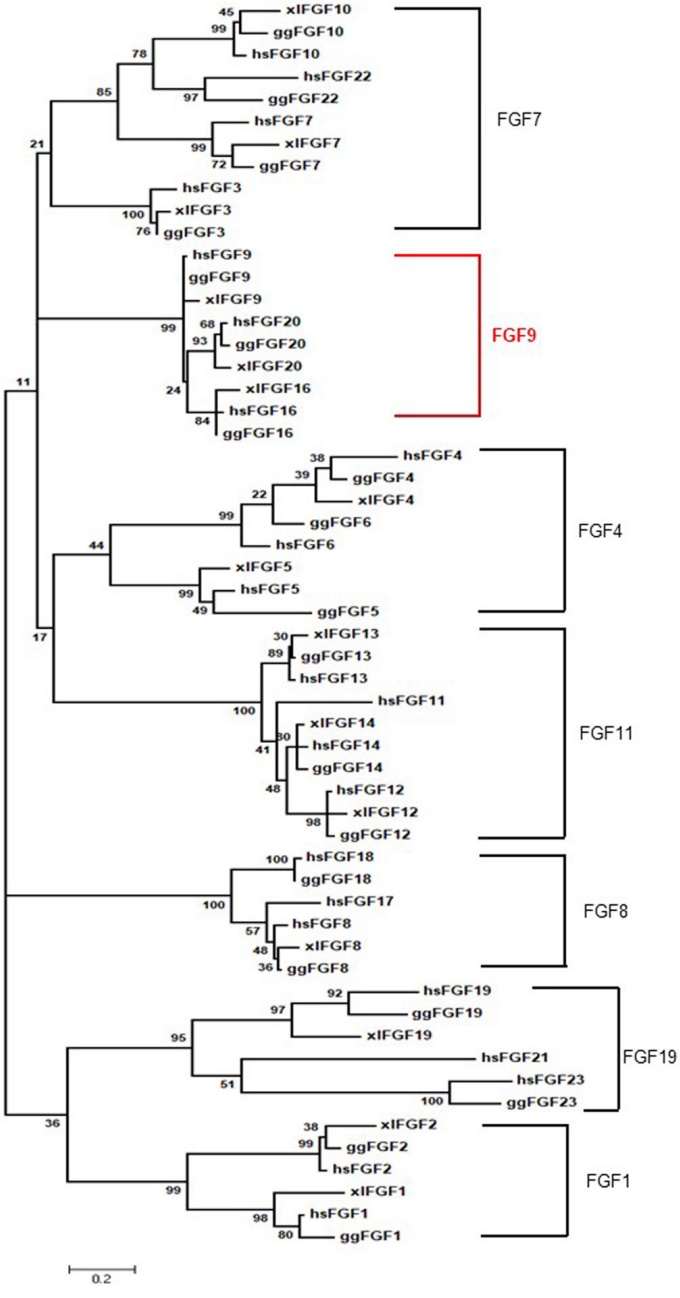

TABLE 1

PRIMER SEQUENCES (5' TO 3')

cdx4	Forward	GGGAGGAATGGAACTCTTATGG
	Reverse	TGTACCGCAGAGTCACAAAG
Egr1	Forward	TCGATCACCTAACAGCAGATG
	Reverse	GGTAGTCTTTGAGACAGGGTATG
Xbra	Forward	GAGCCCACTGGATGAAAGAT
	Reverse	AGCATGTGAAAGAGACGAGTAG
rpl8	Forward	GGGCTRTCGACTTYGCTGAA
	Reverse	ATACGACCACCWCCAGCAA
Sp5	Forward	GCTGTCCTAAGGAATGACTCTC
	Reverse	CTGCACAGAGAGAACACACTT
Sp5l	Forward	ACCTGCATCTTCTGCATCTC
	Reverse	GCCTGAAGATGACAGATATGG
FGF16	Forward	ATGGCTGAGATTGGGAGCGTT
	Reverse	TCACCTATAGTGATAAAGATC

Transcription downstream of FGF signalling has been investigated in *Xenopus* mesoderm (Branney *et al.,* 2009) and the genetic targets identified included *sp5* and *sp5-like* (*sp5l)*, which code for zinc finger transcription factors (Ossipova *et al.,* 2002). Despite this finding in *Xenopus*, most of the knowledge linking *sp5* and *sp5l* to a role downstream of FGF signalling comes from work in zebrafish. *Sp5* (*bts1*) expression in the neural plate was found to be strongly reduced in response to FGF inhibition (Tallafuβ *et al.,* 2001), and *sp5l* expression in the mesoderm is also dependent on the presence of FGF signals (Zhao *et al.*, 2003; Weidinger *et al.*, 2005). Consistent with a role downstream of FGF signalling, Sp5l can posteriorize the neuroectoderm, as it positively regulates posterior neuroectodermal marker *hoxb1b* and represses the anterior markers *fez* and *otx1* in whole embryos (Zhao *et al.*, 2003). These studies support the idea that *sp5* and *sp5l* are downstream targets of FGF signalling in zebrafish. Here we describe the cloning and characterisation of *X. laevis FGF16* and identify *sp5* and *sp5l* as transcriptional targets of FGF signalling during amphibian development.

Results

Cloning of Xenopus laevis FGF16

The predicted sequence for *X. laevis FGF16* on chromosome 8L encodes a 202 amino acid protein that is highly homologous (86% amino acid identity) to human FGF16. The genomic organisation shows syntenic regions in *Xenopus* and human chromosomes, providing confidence that the sequence (GenBank Accession No. XM_018229763.1) encodes for the full-length *X. laevis* FGF16.L protein. PCR primers were designed against the predicted cDNA sequence (Table 1) and a 609bp product was amplified from cDNA derived from *X. laevis* embryos (NF stage 11).

Fig. 1. Phylogenetic analysis of predicted FGF families. *FGF family groupings are represented by brackets. Bold red brackets indicate the FGF9 subfamily: FGF9, FGF16 and FGF20. Protein sequences were aligned using MUSCLE. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jones-Taylor-Thornton (JTT) matrix-based model (Jones* et al.*, 1992). The percentage of trees in which the associated taxa clustered together is shown next to the branches (500 bootstrap support). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Labels are as follows: xl =* Xenopus laevis*; gg =* Gallus gallus*; hs =* Homo sapiens.

Analysis of gene expression at gastrula stages

DIG-labelled antisense RNA probes were used for *in situ* hybridisation analysis of *FGF16* gene expression (Figs 2 and 3). *FGF16* expression is restricted to the dorsal blastopore lip during stage 10, and as gastrulation proceeds, the expression of *FGF16* extends to the mesoderm around the whole of the blastopore by stage 11 and is seen in the posterior mesoderm around the closed blastopore at stage 12. We have analysed the genes *sp5* and *sp5l* as potential targets of FGF signalling; at gastrula stages, both *sp5* and *sp5l* have much wider expression domains than *FGF16* (Fig. 2 D-E and G-H). At NF stage 12, *FGF16*, *sp5* and *sp5l* are all co-expressed in the posterior mesoderm around the closed blastopore (Fig. 2 C, F, and I; arrow).

Analysis of FGF16 at later stages

Using *in situ* hybridisation we have found that *FGF16* is expressed in the posterior mesoderm of early tailbud embryos, as well as the otic vesicle and anterior pituitary (Fig. 3A,B). *FGF16* expression is detected in the branchial arches and mesoderm of later tailbuds (Fig. 3C,D). In the tail, *FGF16* expression is restricted to the chordoneural hinge and the posterior wall of the neuroenteric canal (Fig. 3D) (Tucker & Slack, 1995). *FGF16* is expressed in the anterior pituitary during early tailbud development (Fig. 3B) in *X.laevis*, consistent with findings in zebrafish (Miyake *et al.,* 2014).

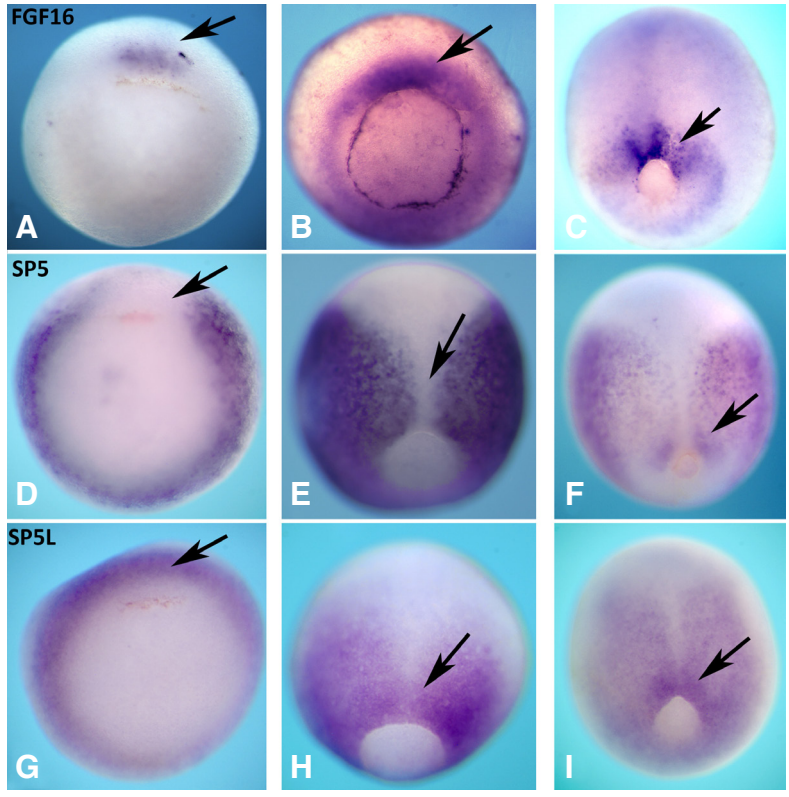

Fig. 2. Expression analysis *FGF16, sp5* and *sp5l* in gastrula stage *X.laevis* embryos. In situ *hybridisation analysis was used to detect transcripts for FGF16* **(A-C)**, *sp5* **(D-F)**, *and sp5l* **(G-I)** *during gastrulation. Stages 10 posterior/vegetal (A, D, G) 11 posterior/vegetal view (B, E, H) and 12 (C, F, I) posterior view. Arrow indicates dorsal blastopore lip in all panels.*

Analysis of sp5 and sp5l in X.tropicalis embryos

The expression of *sp5* and *sp5l* has been described in *X. laevis*, but not in *X. tropicalis*, which we report here (Ossipova *et al.,* 2002). *Sp5* expression in *X. tropicalis* at neurula stage 18 is found in the midbrain and the neural crest, as well as being faintly expressed towards the posterior of the embryo (Fig. 4 A-B). *Sp5l* is expressed along the neural folds and in the posterior region of the embryo (Fig. 4 D-F) and also expressed in migrating crest cells in the branchial arch region (Fig. 4 D). At stage 25, early tailbud, *sp5* has clear expression in the head, including the forebrain, midbrain, midbrain-hindbrain barrier (MHB) and otic placode, but also in a small domain in the tailbud (Fig. 4 C). *Sp5l* at stage 26 is expressed in the posterior of the neural tube and the tailbud, but also in a small domain in the head (Fig. 4G-H). At stage 31, *sp5* expression in *X. tropicalis* is found in the forebrain, midbrain, MHB, branchial arches (BA) 1-4 as previously described in *X.laevis* (Square *et al.,* 2015), and dorsal to the otic vesicles and in the tailbud (Fig. 4 I-K). At this stage, *sp5l* is only expressed in the tailbud (Fig. 4 L-M). These data reveal that the expression patterns of *sp5* and *sp5l* are distinct throughout development and have some overlapping regions of expression to *FGF16*, as well as other FGF ligands (Lea *et al.,* 2009).

Analysis of FGF16 signal transduction

The N-terminus of most FGF ligands has a region of high hydrophobicity called a signal sequence that is required for secretion. However, vertebrate orthologues of FGF9, FGF16 and FGF20

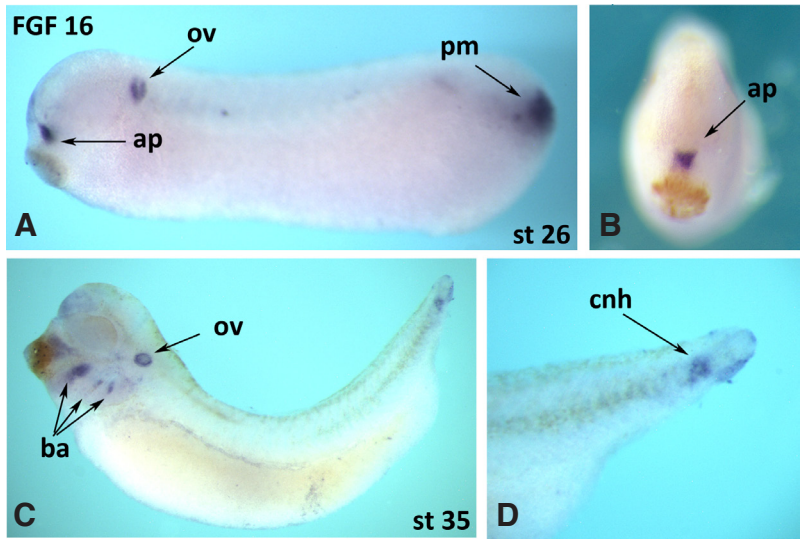

Fig. 3. Developmental expression pattern of FGF16 in *X.laevis* embryos. In situ *hybridisation analysis was used to detect transcripts for FGF16 at several developmental stages in* X. laevis *embryos. At least 15 embryos were analysed at each stage (st).* **(A,B)** *St 26, lateral and anterior views respectively.* **(C,D)** *St 35, lateral and magnified lateral tail views respectively. Abbreviations are as follows: anterior pituitary (ap), otic vesicle (ov), plate mesoderm (pm), branchial arches (ba) and chordoneural hinge (cnh).*

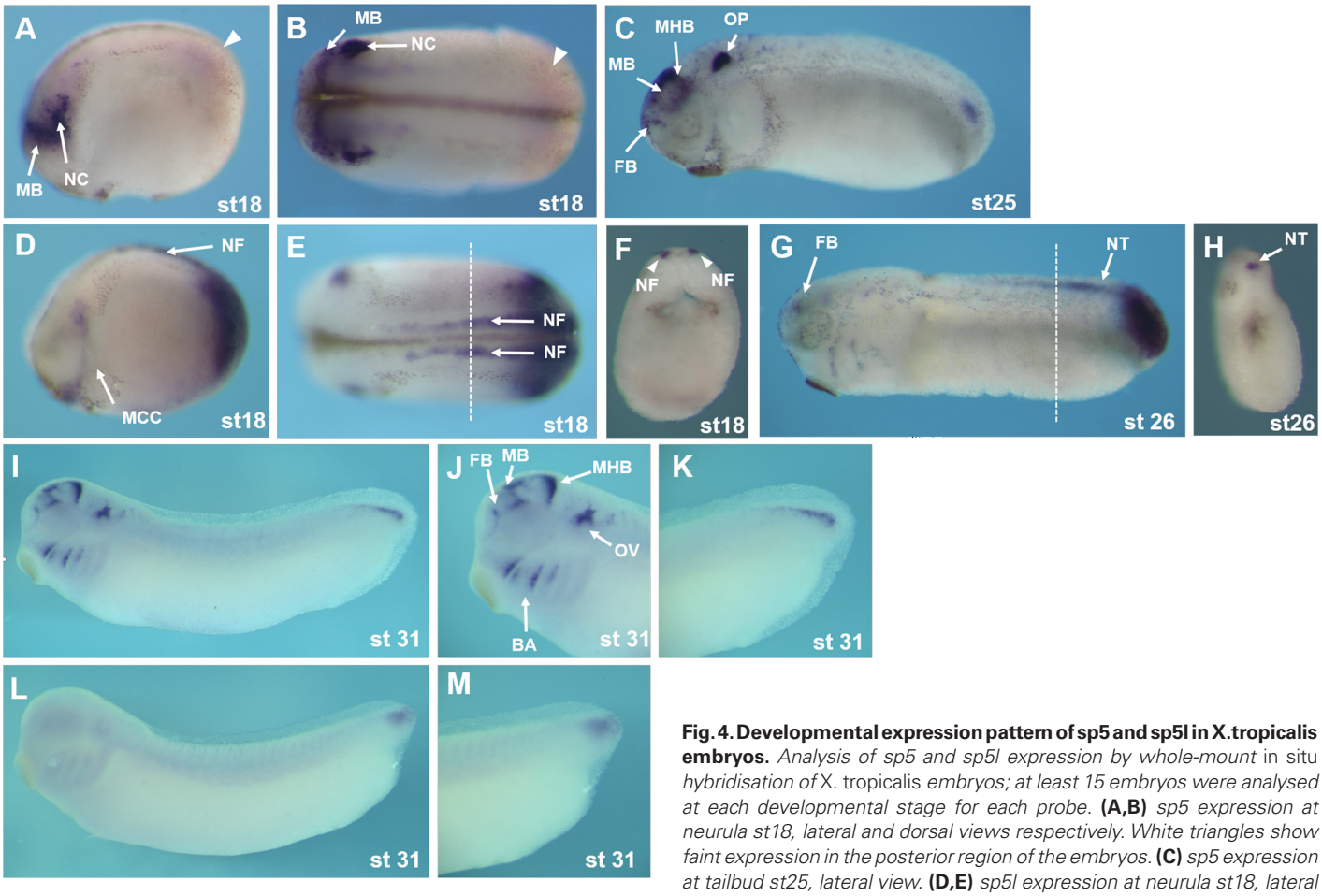

Fig. 4. Developmental expression pattern of sp5 and sp5l in X.tropicalis embryos. *Analysis of sp5 and sp5l expression by whole-mount* in situ *hybridisation of* X. tropicalis *embryos; at least 15 embryos were analysed at each developmental stage for each probe.* **(A,B)** *sp5 expression at neurula st18, lateral and dorsal views respectively. White triangles show faint expression in the posterior region of the embryos.* **(C)** *sp5 expression at tailbud st25, lateral view.* **(D,E)** *sp5l expression at neurula st18, lateral and dorsal views respectively.* **(F)** *Transverse section of the embryo in (E) at the level of the dashed line.* **(G)** *sp5l expression at tailbud st26, lateral view.* **(H)** *Transverse section of embryo in (G) at the level of the dashed line.* **(I-K)** *sp5 expression at tailbud st31, lateral view.* **(L,M)** *sp5l expression at tailbud st31, lateral view. Abbreviations: Midbrain (MB), Neural crest (NC), Forebrain (FB), Otic placode (OP), Midbrain Hindbrain Boundary (MHB), Migrating crest cells (MCC) Neural fold (NF), Neural tube (NT), Branchial Arches (BA), Otic Vesicle (OV).*

have divergent N-termini and lack a signal sequence (Miyakawa & Imamura, 2003). These FGF ligands are characterised by conserved mid-regions and C-termini that allow secretion (Katoh & Katoh, 2005). This is shown in Fig. 5, where the low N-terminus hydrophobicity for the FGF9 subfamily is compared to FGF4 (Fig. 5A, compared to Fig. 5 B-D). The FGF9 subfamily members contain an internal hydrophobicity region, which likely aids the proteins' secretion out of the cell (Miyakawa & Imamura, 2003).

The animal cap is a source of pluripotent cells which develops into atypical epidermis in the absence of additional signals (Green, 1999). Upon treatment with growth factors, including FGFs, the animal cap can be diverted from this epidermal fate to differentiate into different tissue types, such as mesoderm (Kimelman & Kirschner, 1987; Slack *et al.*, 1987). Therefore, this approach provides a robust biological assay for FGF16 activity (Fig. 6). ERK, also known as MAPK, is the effector of the Ras-Raf-MEK-MAPK signalling pathway. The diphosphorylation of ERK (dp-ERK) indicates that FGF16 can strongly activate the MAPK signalling pathway in animal caps (Fig. 6A). FGF16 is shown to affect cell behaviour, due to untreated animal caps forming round balls of atypical epidermis after 3 days (Fig. 6B), whereas those expressing FGF16 form vesicles of mesoderm including blood, mesothelium and muscle (Fig. 6C).

FGF signalling activates sp5 and sp5l expression

Previous research suggests that *X.laevis sp5* and *sp5l* are positively regulated targets of FGF signalling (Branney *et al.*, 2009; Park *et al.*, 2013). To further test this possibility, the effect of increasing FGF signalling on *sp5* and *sp5l* expression was investigated. *X. laevis* embryos were injected with 10pg of mRNA coding for *FGF4*, known to be a potent mesoderm inducer (Isaacs *et al.*, 1994), or 50pg of mRNA coding for *FGF16*. RT-PCR was performed on stage 12 whole embryos and animal cap explants from injected embryos compared to uninjected controls. Expression of *sp5*, *sp5l* and known FGF targets, *xbra*, *cdx4*, and *gr1*, was analysed. Both *FGF4* and *FGF16* injection induced the FGF target genes as well as *sp5* and *sp5l* (Fig. 6D).

Collectively these results show that *sp5* and *sp5l* are downstream targets of FGF signalling, with the identification of FGF16 as a novel ligand that has mesoderm inducing activity.

Discussion

FGF16 signal transduction

In this study, FGF16 was found to be able to activate MAPK signalling and induce mesoderm. The Ras-Raf-MEK-ERK (MAPK)-

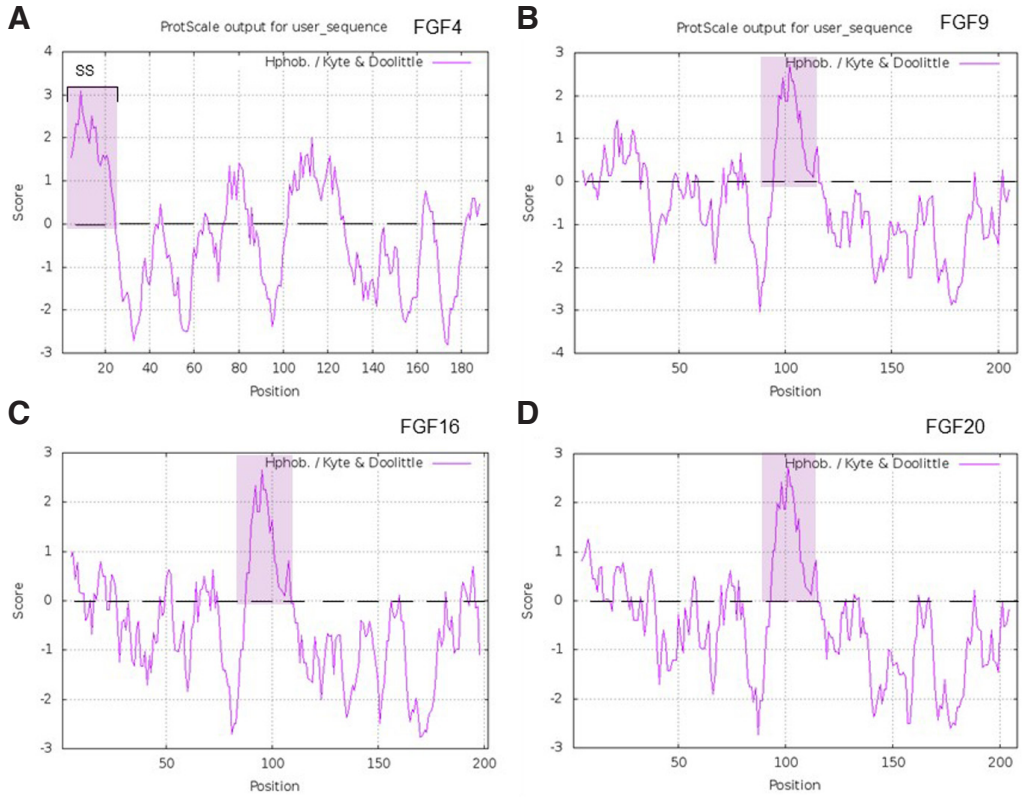

Fig. 5. Hydrophobicity plots for FGF9 family. *Kyte and Doolittle hydrophobicity plots for* **(A)** *FGF4 and FGF9 sub-family members,* **(B)** *FGF9,* **(C)** *FGF16, and* **(D)** *FGF20. Scores above 0 (represented by the dotted line) indicate hydrophobic amino acids; sites of high hydrophobicity are depicted as shaded areas. FGF4 has a "classic" signal sequence (SS) for co-translational secretion, but the members of the FGF9 subfamily have an internal hydrophobicity region which is associated with secretion.*

pathway has a well-defined role in regulating mesoderm induction in response to FGF signalling (Cornell & Kimelman, 1994; LaBonne *et al.*, 1995; LaBonne & Whitman, 1994; Umbhauer *et al.*, 1995; Whitman & Melton, 1992). FGF16 has also been shown to signal through the MAPK pathway in human ovarian cancer cells (Basu *et al.*, 2014), and here we demonstrate FGF16-mediated MAPK activity to be observed in the mesoderm induction assay. The role of FGF9, FGF16 and FGF20 in mesoderm induction has not been well characterised. However, *FGF9/16/20*, an ancestral form of the vertebrate FGF9 subfamily, has been shown to induce mesenchyme formation in *Ciona intestinalis* embryos (Imai *et al.*, 2002; Tokuoka *et al.*, 2004). The presence and activity of this class of FGF ligand in a distant chordate relative supports the notion that FG9/16/20 is a conserved factor for mesoderm specification (Davidson *et al.*, 2006). Furthermore, an RNA-seq screen of *X. laevis* gastrula expression in dorsal and ventral lip tissue identified *FGF16.S* and *FGF16.L* as having moderate positive Pearson correlation coefficients of 0.48 and 0.25 respectively to chordin, indicating the two genes may share transcriptional regulation mechanisms and that FGF16 may be involved in dorsal-ventral patterning (Ding *et al.*, 2017).

The paracrine FGFs signal through the four different FGFRs, triggering multiple downstream signalling pathways to result in the regulation of transcription factors required for controlling many different developmental processes, such as mesoderm induction (reviewed in Ornitz and Itoh, 2015). The alternative splicing of FGFR receptors greatly increases ligand binding specificity, particularly through the generation of two isoforms of Ig-like domain III (epithelial b splice forms or mesenchymal c splice forms) (Yeh *et al.*, 2003). FGF9 subfamily members have similar FGFR binding affinities (Zhang *et al.*, 2006). For example, mouse homologue FGF9 preferentially binds to FGFR2 and FGFR3 c splice variants, displaying greatest affinity for FGFR3, but does not bind FGFR1 or FGFR4 (Hecht *et al.*, 1995; Ornitz *et al.*, 1996; Santos-Ocampo *et al.*, 1996). Zhang *et al.,* (2006) confirm a strong binding of FGF9, 16 and 20 to FGFR3c, FGFR3b and FGFR2c when cataloguing the receptor binding of all FGF ligands in the murine Baf3 cell line. FGF20 binding matches the other FGF9 subfamily members, although it has higher affinity for FGFR2b (Zhang *et al.*, 2006). Murine FGF16 appears to have the highest affinity for FGFR3c, followed by FGFR3b and FGFR2c, but does not bind to FGFR2b or FGFR1b and only weakly binds FGFR4 (Zhang *et al.*, 2006). The same binding profile for FGF16 is revealed using the Baf3 assay, with very weak FGFR1c affinity demonstrated (Lavine *et al.*, 2005; Lu *et al.*, 2008). Furthermore, FGF16-mediated invasion in a human ovarian cancer model persists upon addition of a selective FGFR1 inhibitor, PD 173074 (Mohammadi *et al.*, 1998), confirming FGF16 to not preferentially signal through FGFR1 (Basu *et al.*, 2014). Konishi *et al.,* (2000) also report that FGF16 only binds the extracellular domain of FGFR4, showing no affinity for FGFR1c or FGFR2c, during embryonic brown adipose tissue development, suggesting altered FGF16 ligand specificity for different developmental stages or tissue types.

FGF16 secretion

Phylogenetic analysis of sequences obtained from Xenbase matched the consensus arrangement of the 22 FGF members into 7 different subfamilies (Fig. 1) (Itoh & Ornitz, 2004). FGF9 subfamily members are highly homologous in structure and have similar receptor binding sites (Itoh & Ornitz, 2004; Zhang *et al.*, 2006). For example, they have a conserved C-terminus and central hydrophobic region, which is required for secretion (Miyakawa &

Imamura, 2003). Despite this knowledge, the mechanism of FGF16 secretion remains to be elucidated.

Most FGFs require N-terminal signal peptides for secretion (Kapp *et al.*, 2009). However, FGF1, FGF2, FGF9, FGF16 and FGF20 lack cleavable signal peptides. FGF1 and 2 can be released in response to damage via an ER-Golgi independent exocytotic secretory pathway (Itoh & Ornitz, 2004; Mignatti *et al.,* 1992). Instead, FGF2 directly translocates across the plasma membrane for secretion (Nickel, 2010; Zehe *et al.*, 2006). Although FGF9 subfamily members are secreted, they reply upon uncleavable bipartite signals including the N-terminal and central hydrophobic region (Miyakawa *et al.*, 1999; Revest *et al.*, 2000). Interestingly, Miyakawa & Imamura (2003) showed that FGF16 is secreted by a process requiring the endoplasmic reticulum and Golgi; retrograde Golgi transport of FGF16 was first inhibited by using brefeldin A and N-glycosylation of the N-terminus was separately observed, indicating that FGF16 must have progressed to the ER for processing.

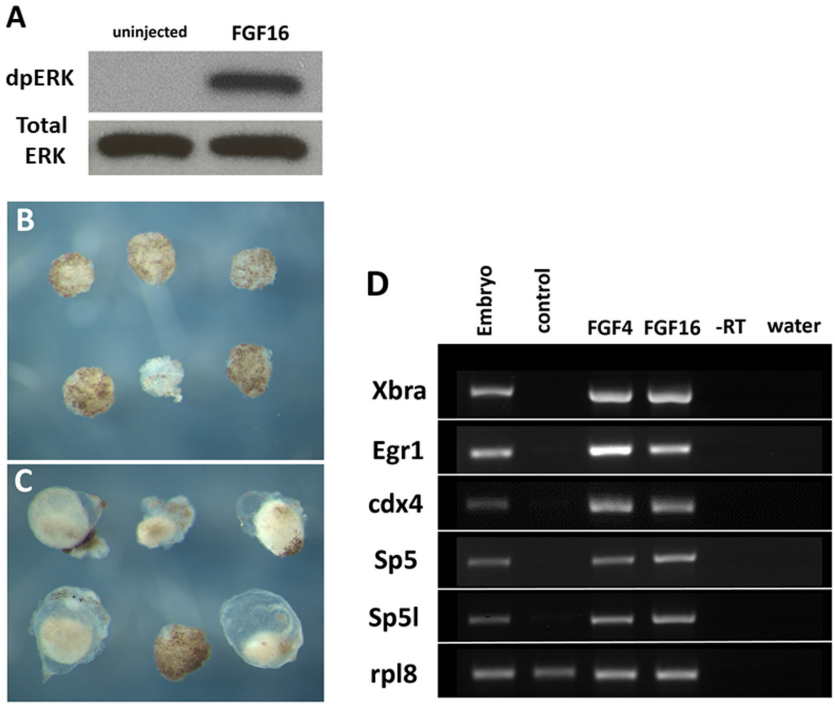

Fig. 6. FGF16 activates the MAPK pathway and activates sp5 and sp5l expression. **(A)** *Western blot to detect dpERK in uninjected control animal caps compared and animal caps injected with 50 pg of mRNA coding for* FGF16 *mRNA. Antibodies to diphospho-ERK (dp-ERK) and total ERK were incubated on the same blot sequentially after stripping.* **(B)** *Control animal caps after 3 days culture.* **(C)** *Animal caps expressing FGF16 from sibling embryos to those shown in (B).* **(D)** *RT-PCR on cDNA derived from whole embryos at stage 12 and on animal cap explants cultured to stage 12 that were either uninjected control explants or explants expressing* FGF4 *or* FGF16. *Expression of known FGF targets,* xbra *(27 cycles),* egr1 *(27 cycles)* and cdx4 *(27 cycles), as well as* sp5 *(25 cycles),* sp5l *and* rpl8 *loading control. Water, in which* H_2O *replaced cDNA, and - RT, where no reverse transcriptase was used, functioned as negative controls.*

sp5 and sp5l expression and regulation

Our data indicate that *sp5* and *sp5l* are candidates for regulation by FGF16. There is overlap of their expression domains in the early mesoderm and FGF16 is able to activate the expression of both *sp5* and *sp5l* in a mesoderm induction assay. Compared to its relatives, *sp5* is unique having two paralogs in the *Xenopus* and teleost lineages (Ossipova *et al.,* 2002; Zhao *et al.*, 2003, Tallafuβ *et al.*, 2001), whilst there is only one locus present in mammals (Harrison *et al.*, 2000). Phylogeny suggests that these genes were duplicated from the same ancestor, but one copy was lost subsequently from the mammalian and bird lineages in evolution (Zhao *et al.*, 2003; Pei and Grishin, 2015). In *Xenopus* these paralogs are referred to as *sp5* and sp5l.

The Sp1-like transcription factors are a family of proteins with important regulatory roles in development (Reviewed by Zhao and Meng., 2005). The family comprises of Sp1 along with a number of structurally similar transcription factors which share defining features such as a triple C_2H_2 zinc finger domain in the C-terminal region and a preceding buttonhead (Btd) box (Reviewed by Zhao and Meng, 2005; Ossipova *et al.*, 2002). Sp1-like transcription factors have been identified in a variety of species, including *Xenopus,* which has 10 members (Presnell *et al.*, 2015). These transcription factors regulate the expression of target genes by acting as transcriptional activators or repressors in a context-dependent manner (Fujimura *et al.*, 2007; Hagen *et al.*, 1995; Birnbaum *et al.*, 1995; Phan *et al.*, 2004; Majello *et al.*, 1997). The DNA binding specificity of these factors is similar across the family, with the conserved triple zinc finger domain recognising GC-rich sequences in the promoter regions of genes (Kadonaga *et al.*, 1987). Differences in key residues of the zinc finger motifs modulate this DNA binding specificity between family members (Reviewed by Kaczynski *et al.*, 2003). Although this family shares commonality in structure and DNA binding, different members appear to play distinct roles in embryonic development, which is demonstrated by their dynamic expression patterns (Reviewed by Zhao and Meng, 2005). *In situ* hybridisation in *Xenopus* species showed that *sp5* and *sp5l* have distinct expression patterns throughout development.

Sp5 and *sp5l* clearly have differential expression patterns throughout development, indicating they likely have different functional roles. Presence of *sp5* in the neural crest and related structures, the branchial arches and otic vesicle, suggests a role in neural crest formation. Supporting this hypothesis, knockdown of *sp5* results in defective neural crest structures and *sp5* overexpression or loss causes up- or down-regulation of neural crest markers *sox10*, *sox9* and *slug* (Park *et al.*, 2013). *Sp5l* expression is similar to posterior factors *cdx4* and *hoxA7*, with expression in the posterior neural tube and tailbud (Northrop and Kimelman, 1993; Pownall *et al.*, 1998) suggesting a role in posterior patterning.

In comparison to *Xenopus*, murine *Sp5* is expressed in the primitive streak throughout gastrulation and is subsequently in the midbrain, MHB, neural tube, somites, pharyngeal region and the tailbud (Harrison *et al.*, 2000). *Xenopus sp5* and *sp5l* recapitulate different aspects of mammalian *Sp5* patterning and possibly function. Murine *Sp5* and *Xenopus sp5* share expression in the midbrain, MHB and pharyngeal region, whereas *Xenopus sp5l* and murine *Sp5* share expression in the neural tube. Both

Xenopus paralogs are expressed in the tailbud, like murine *Sp5*. Mammals do not possess *Sp5l*, hence mammalian *Sp5* may perform the roles of both *Xenopus sp5* and *sp5l*. This suggests a divergence of function in *Xenopus* Sp5 and Sp5l proteins after their duplication event, whilst mammals lost their *Sp5l* locus and Sp5 retained all function.

Materials and Methods

Embryos

X. laevis and *Xenopus tropicalis* embryos were obtained using artificial fertilisation. *X.laevis* embryos were microinjected in 0.3 x normal amphibian medium (NAM) + 5% ficoll and cultured in 0.1 x NAM between temperatures of 14°C – 23°C. *X.tropicalis* embryos were cultured in MRS/20 between temperatures 20°C – 24°C. All embryos were staged according to Nieuwkoop & Faber (1967).

Multiple sequence alignments for FGF16

Xenbase was used to obtain the available *X. laevis* FGF amino acid and coding sequences (Karimi *et al*., 2018). BLAST (Basic Local Alignment Search Tool) searches identified the *Gallus gallus* (chick) and *Homo sapiens* (human) FGF sequences (Priyam *et al*., 2015). *X. laevis* FGF16 is unavailable on Xenbase; the human FGF16 amino acid sequence was used to identify the *X. laevis FGF16* genomic sequence. The apparent evolutionary relationship between all members of the *X. laevis, Gallus gallus* and *Homo sapiens* FGF families was examined. A maximum-likelihood phylogenetic tree was created using Mega7 software and alignments performed for amino acid sequences using MUSCLE (Edgar, 2004; Kumar *et al*., 2016). FGF9, FGF16 and FGF20 alignments were created using GeneDoc software (Nicholas, 1997).

Cloning of Xenopus laevis FGF16

RNA was extracted from stage 11, 17, 20 and 25 *X. laevis* embryos via Trizol (Invitrogen) extraction and the Zymo-Spin™ IC column RNA preparation procedure (Zymo Research), according to the manufacturer's instructions. Extracted RNA was used to generate cDNA using the SuperScript® IV First-Strand Synthesis System (Invitrogen). Stage 11 cDNA was amplified by polymerase chain reaction (PCR) with GoTaq® Green Master Mix (Promega) and specific FGF16 primers (Table 1). PCR products were ligated into the pGEM-T Easy DNA vector (Promega), and subsequently subcloned into pCS2+ using EcoR1.

In vitro transcription of DIG-labelled probes

pGEM-*FGF16* was linearized using Sph1; pCS107 containing *Sp5* (GenBank Accession: AAH62500) using NcoI, and pCS107 containing *Sp5l* (GenBank Accession: AAI54679) using HindIII before transcription using the DIG RNA Labelling Kit (Roche). T7 RNA polymerase was used for *sp5 and sp5l* DIG transcription and SP6 RNA polymerase for *FGF16*.

In situ hybridisation

Embryos were collected at the appropriate stages and fixed in MEMFA (0.1M MOPS, 2mM EDTA, 1mM $MgSO_4$, 3.7% formaldehyde). *In situ* hybridisations were performed following the procedure described in Fisher *et al., (*2002). Hybridisation was carried out overnight at 60°C with 1μg/ml of *FGF8*- and *FGF16*- DIG probes. Embryos were then incubated overnight at 4°C with a 1/2000 dilution of affinity-purified sheep anti-DIG antibody coupled to alkaline phosphatase (AP) in blocking solution. Colour reactions were subsequently performed overnight using the BM purple precipitating AP detection system (Roche). Pigment was removed using 5% H_2O_2 solution in PBS.

Photography

Images were taken using the SPOT insight 4 MP CCD colour camera attached to a Leica MZFLIII microscope.

Generating synthetic mRNA

pCS2+-*FGF16* was linearized using NotI and Capped *FGF16* mRNA was generated using the mMessage mMachine SP6 transcription kit (Invitrogen), following the manufacturer's instructions.

Western blot analysis

X. laevis animal cap explants were dissected at NF stage 8 and cultured until NF stage 12 when they were collected and homogenised in sample buffer and centrifuged (Keenan *et al*., 2006). Supernates were loaded onto an acrylamide gel for SDS-PAGE and protein electro-transferred onto Immobilon-P PDVF membranes (Millipore). Membranes were blocked in 5% milk/PBSAT. Primary antibody dilutions were: mouse α-dp-ERK (Sigma), 1/4000; α-total-ERK, 1/500,000; α-pP38, 1/2000; α-pAKT, 1/5000. Secondary antibody dilutions were: dpERK α-mouse HRP, 1/4000; total ERK, pP38 and pAKT α-rabbit, 1/2000. BM chemiluminescence blotting substrate (Roche) was used for peroxidase activity detection.

Semi-quantitative RT-PCR

5 embryos, or 10 animal cap (ectodermal) explants, were flash-frozen on dry ice, and RNA was extracted using TRIzol Reagent® (Sigma). cDNA was synthesised from 1μg of total RNA using SuperScript® IV Reverse Transcriptase (Invitrogen). In short, total RNA was incubated for 5mins at 65°C with 50μM random hexamer primers and then incubated with reverse transcriptase at 23°C for 10mins, 55°C for 10mins and 80°C for 10mins. To check for any genomic contamination, control RNA was also processed without reverse transcriptase. PCR amplification was performed using primers in Table 1, including those for *ribosomal protein L8* (*rpl8*) as a ubiquitously expressed loading control.

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