# Supporting Information for

# The cytokine FAM3B/PANDER is an FGFR ligand that promotes posterior development in *Xenopus*

**Fangfang Zhang[a,b,1], Xuechen Zhu[c,d,1], Pan Wang[e,f,1], Qing He[a,b], Huimei Huang[g], Tianrui Zheng[f], Yongyu Li[f], Hong Jia[b], Linping Xu[b], Huaxiang Zhao[h], Gabriele Colozza[i], Qinghua Tao[d,f,2], Edward M. De Robertis[i,2] and Yi Ding [a,b,2]**

[1]F.Z., X.Z., and P.W. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: qhtaolab@mail.tsinghua.edu.cn, ederobertis@mednet.ucla.edu, or dingyi1510@xjtu.edu.cn.

**This PDF file includes:**

SI Materials and Methods

Figs. S1-S5

SI References

**SI Materials and Methods**

**Additional Cloning.** Human FAM3B mutant (C91A/C229A, disulfide bridges formation defective) was generated using a QuikChange Site-Directed Mutagenesis Kit (Agilent, catalog no. 200522) with pCS2-*FAM3B-HA* as template. pCS2-*FAM3B-His* was generated by performing PCR with primers containing 6 repetitive histidine codons right after Gly-Ser-Gly (GSG) linker sequences using human FAM3B cDNA as template and the PCR primers were:

| | |
|---|---|
| Forward | 5' GGCATCGATACCATGCGCCCATTGGCTGGT 3' |
| Reverse | 5' GGCGATATCTTAGTGGTGATGGTGATGATGTCCGCTTCC GCTTCGTTCTTTGGGTATGCAGCC 3' (underlining indicates 6×His and GSG linker sequences) |

pcDNA6.2-emGFP-FGFR1 and pcDNA6.2-emGFP-FGFR1-KI (Y653F/Y654F, kinase-inactive) were kindly provided by Prof. Daniela Rotin in University of Toronto. pCS2-*FGFR1-HA* and pCS2-*FGFR1-KI-HA* were generated by subcloning human FGFR1 and FGFR1-KI into pCS2 vectors with carboxy-terminal HA Flag. pCS2-*FGFR1-Ecto-3×Flag* was generated by cloning the ectodomain of human FGFR1 (1-376 aa; NP_075598.2) into pCS2 vectors with carboxy-terminal Flag tag. pCMV-Klb-EGFP was a gift from Prof. Zipora Yablonka-Reuveni (Addgene plasmid # 45531) (1) and pCS107-*β-Klotho-Ecto-HA* was generated by subcloning the ectodomain of mouse Klb (β-Klotho) (1-994 aa; NP_112457.1) into pCS107 vector with carboxy-terminal HA tag. The FGF reporter was generated by cloning a fragment of 508 bp of mouse *Dusp6* promoter region (-863 bp to -355 bp; XM_036155965.1) into pGL3-Basci vector.

**Whole Mount In Situ Hybridization**. WISH were performed following standard protocols as previously described (2). The following hybridization probes from the De Robertis lab were used: *gsc*, *bix4*, *bf1*, *otx2*, *rx2a*, *hoxb9*, *xpo*, *xbra*, *fgf3*, *cdx4*, *sox2*, *chd*, *not*, *myod* and *fam3b*. For the *fam3b* probe, a 1.5 kb PCR fragment was amplified from cDNA of stage 13 embryos with the following primers:

| | |
|---|---|
| Forward | 5' GCTTCCAGACTGACACAATG 3' |
| Reverse | 5' AATTAACCCTCACTAAAGGGGGGAATTGGGAACAAATGGG 3'<br>(underlining indicates the T3 promoter used for RNA transcription) |

The PCR product was then purified with a PureLink purification kit (Invitrogen, catalog no. K3100-01) and used as a template to synthesize the antisense probe using T3 RNA polymerase as described (3).

**RT-PCR**. RT-PCR experiments on *Xenopus* embryos were performed as previously described (2). Primer sequences for RT-PCR are as follows:

| | |
|---|---|
| *Xenopus fam3b.l* Forward | 5' CTGTTTGCAGAGGTATTG 3' |
| *Xenopus fam3b.l* Reverse | 5' GTAGGCAAGTTGGTTTG 3' |
| *Xenopus xbra* Forward | 5' TTCTGAAGGTGAGCATGTCG 3' |
| *Xenopus xbra* Reverse | 5' GTTTGACTTTGCTAAAAGAGACAGG 3' |
| *Xenopus odc* Forward | 5' GCCATTGTGAAGACTCTCTCCATTC 3' |
| *Xenopus odc* Reverse | 5' TTCGGGTGATTCCTTGCCAC 3' |

**Animal Cap Assays.** Animal caps (ectodermal explants) were excised from stage 8 embryos and cultured in 1× MMR in the presence of recombinant human FAM3B-His protein or human FGF2 protein (Sino Biological, catalog no. 10014-HNAE) or human FGF8B protein (Sino Biological, catalog no. 16277-HNAE) with concentrations indicated in figure legends. Animal caps were cultured until stage 11 for RT-PCT with the mesodermal marker *xbra* or stage 16 for assessment of elongation. Data shown are representative of at least three independent experiments.

**Protein Sequence Alignments.** Sequences of human FAM3B, mouse FAM3B and *X. laevis* Fam3b were aligned using the Clustal Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/). These alignments were then further analyzed with Boxshade (https://embnet.vital-it.ch/software/BOX_form.html), a program that assigns color shades to indicate how similar two sequences are. Black shading indicates residue identity on that position, while grey shading indicates residue similarity.

**Cell Culture and Transfection.** HEK293T cells were cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO, catalog no. 10270-106), 1% penicillin/streptomycin, and 1% glutamine. The cells were cultured at 37 °C and 5% $CO_2$. DNA transfections were performed with Lipofectamine™ 3000 (Invitrogen, catalog no. L3000-015) following manufacturer's instructions.

**Conditioned Medium Preparation and Secretion Assay.** For human FAM3B conditioned medium (CM) preparation, 10 cm plates of HEK293T cells were transfected with pCS2-*FAM3B-His* using Polyethylenimine (PEI) (Polysciences, catalog no. 23966-1). 24 hours later, the cells were washed with PBS for 3 times to remove residue serum and culture medium was replaced with 6 ml of secretion medium (1/3 Iscove's, 2/3 DMEM/F12 (1:1), 100 U/ml Penicillin, 100 µg/ml

Streptomycin, 2 mM L-Glutamine, 1× MEM NEAA). 30 hours later conditioned media were collected and centrifuged to remove cell debris and the pH was adjusted to 7.4. Conditioned media of negative control (derived from pCS2 empty vector transfected HEK293T cells), FAM3B-HA, FAM3B-Flag, FGFR1-Ecto-Flag and β-Klotho-Ecto-HA were generated as described above. For human FAM3B and *Xenopus* Fam3b secretion assay in HEK293T cells, conditioned media were concentrated 5 times using Amicon Ultra centrifugal filter-10K (Millipore, catalog no. UFC501024) following manufacturer's indications and analyzed by Western blot.

**Western blot.** Cells and *Xenopus* embryos were lysed with TNE lysis buffer (10 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) together with protease inhibitors (Roche, catalog no. 04693132001) and phosphatase inhibitors (Calbiochem, catalog no. 524629). Western blots were performed using standard protocols. Blotted nitrocellulose membranes were analyzed using the LI-COR Odyssey System. Alternatively, protein bands were detected by ECL chromogenic kit (Huaxingbio, catalog no. HXP1868), and images were visualized with the Tanon-5200 chemiluminescence imaging system (Tanon Science & Technology, Shanghai, China). The following primary antibodies and dilutions were used: rabbit anti-HA (Cell Signaling Technology, catalog no. C29F4, 1:1000), mouse M2 anti-Flag (Sigma, catalog no. F3165, 1:5000), mouse anti-His (Origene, catalog no. TA150088, 1:2000), rabbit anti-ERK (Cell Signaling Technology, catalog no. 137F5, 1:2000), rabbit anti-pERK (Cell Signaling Technology, catalog no. 9101, 1:1000), mouse anti-α Tubulin (Calbiochem, catalog no. CP06, 1:3000), mouse anti-α Tubulin (Origene, catalog no. TA503129, 1:3000), rabbit anti-GAPDH (Cell Signaling Technology, catalog no. 2118, 1:3000) and goat anti-Human IgG (Fc specific) antibody (Sigma, catalog no. I2136, 1:1000). For LI-COR Odyssey System, secondary antibodies and dilutions were: donkey anti-rabbit IRDye 800 CW (Li-Cor, catalog no. 926-32212, 1:3000) and donkey anti-mouse IRDye 680

LT (Li-Cor, catalog no. 926-68072, 1:3000). For the Tanon-5200 chemiluminescence imaging system, secondary antibodies and dilutions were: donkey anti-rabbit IgG-HRP (Solarbio, catalog no. SE134, 1:3000) and donkey anti-mouse IgG-HRP (Solarbio, catalog no. SE131, 1:3000).

**Immunoprecipitation.** Conditioned media (serum free) were prepared from HEK293T cells transfected with pCS2-*FAM3B-HA*, pCS2-*FAM3B-3×Flag*, pCS2-*FGFR1-Ecto-3×Flag* or pCS107-*β-Klotho-Ecto-HA* and mixed as indicated. Immunoprecipitation between FAM3B and FGFR1-Ecto or β-Klotho-Ecto was performed with the media using Anti-FLAG M2 Magnetic Beads (Sigma, catalog no. M8823). Except for a small fraction of input, the remaining media was incubated with the beads with rotation for 4 hours at 4°C. In addition, to detect the interaction between FAM3B and full length FGFR1, two-cell stage *Xenopus* embryos were injected after cleavage with *FAM3B* and wild type of kinase-inactive mutant *FGFR1* mRNAs separately into different blastomeres as indicated. Gastrula stage embryos were collected and lysed in TNE lysis buffer together with protease inhibitors and phosphatase inhibitors. Except for a small fraction of input, the remaining supernatant was incubated with the Anti-FLAG M2 Magnetic Beads with rotation for 4 hours at 4°C. In both cases, beads were washed three times and 10 min each. Finally, the protein complex bound to beads were eluted off with 1 mg/ml 3×Flag peptide and denatured in 4× loading buffer at 100 ºC for 5 min followed by SDS-PAGE and Western Blot.

**Luciferase Reporter Assay.** HEK293T cells were transfected with FGF reporter derived from mouse *Dusp6* promoter together with pRL-TK Renilla and FGFR1 KI constructs. 20 hours after transfection, cells were serum starved for 20 hours, followed by treatment with control or human FAM3B CM for 8 hours. After treatment, cells were lysed in Passive Lysis Buffer (Promega) and Luciferase assays were performed with the Dual-Luciferase Reporter Assay System (Promega,

catalog no. E1980) according to manufacturer's instructions, using a Glomax Luminometer (Promega). Renilla readings were used for normalization.

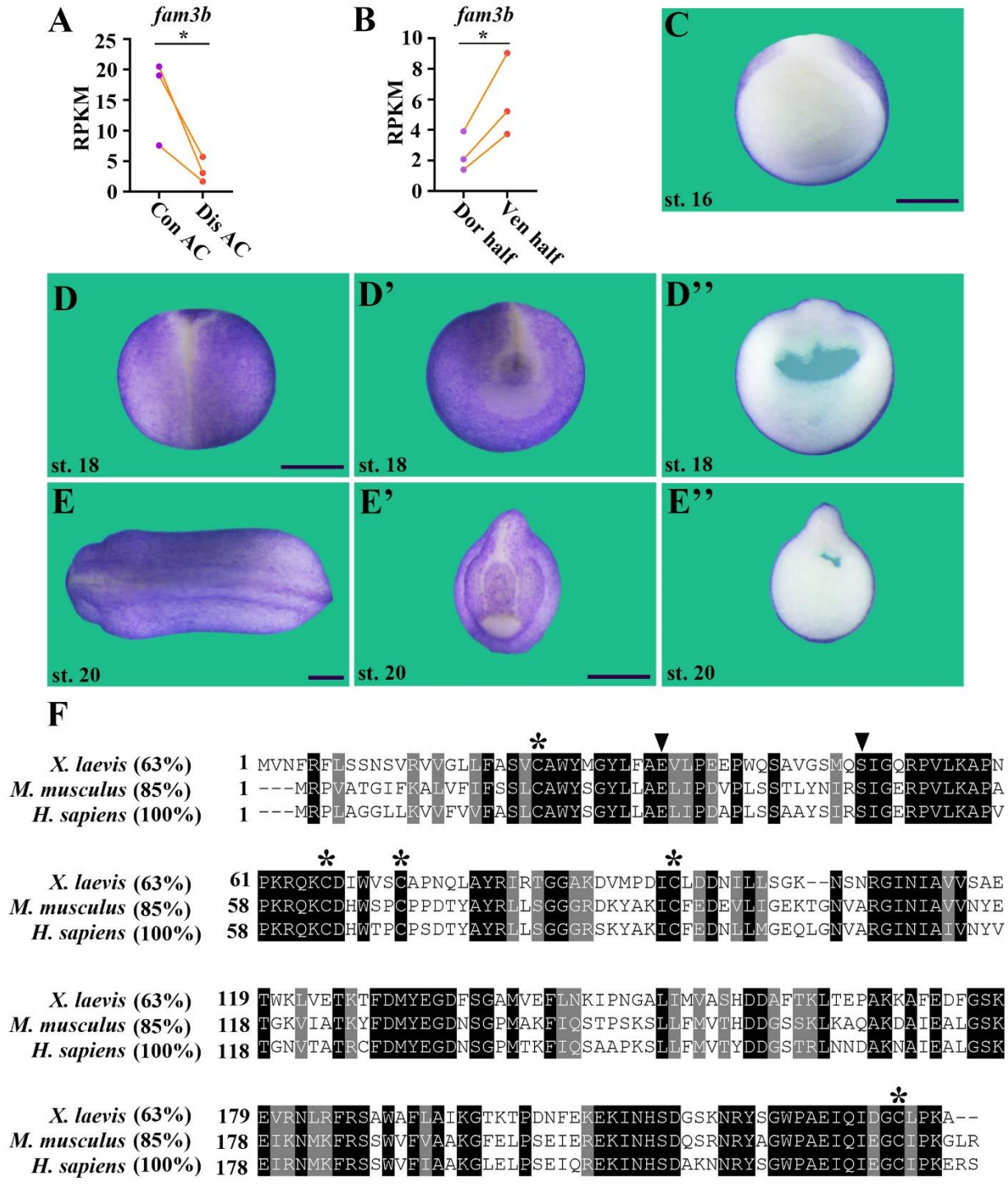


**Fig. S1.** *Xenopus fam3b* encodes an epidermal protein conserved in vertebrates. (*A*) Data plot showing that *fam3b* was identified in genome-wide RNA-seq studies as a putative secreted protein-encoding gene decreased by animal cap dissociation in the absence of $Ca^{2+}$ and $Mg^{2+}$. Animal caps were dissected at stage 9 and cultured until stage 12 with or without cell dissociation followed by genome-wide RNA-seq analysis to identify dissociation-regulated transcripts. Data from three biological replicate experiments are shown. RPKM: Reads per kilo base per million mapped reads.

Con AC: Control animal caps. Dis AC: Dissociated animal caps. Statistical significance was assessed by paired one-tailed Student's t-test. *: $P<0.05$. (*B*) Data plot showing *fam3b* is enriched in ventral halves compared to dorsal halves of *Xenopus* embryos. Embryos were cut into dorsal and ventral halves at stage 10 and cultured until stage 12 followed by genome-wide RNA-seq analysis to identify dorsally or ventrally enriched transcripts. Data from three biological replicate experiments are shown. Dor half: Dorsal half. Ven half: Ventral half. Statistical significance was assessed by paired one-tailed Student's t-test. *: $P<0.05$. (*C*) Cross section of stage 16 embryos showing *fam3b* expression in the epidermis. (*D-D"*) WISH of *fam3b* showing its expression in the epidermis at stage 18. *D*, dorsal view; *D'*, anterior view; *D"*, cross section. (*E-E"*) WISH of *fam3b* showing its expression in the epidermis at stage 20. *E*, dorsal view; *E'*, anterior view; *E"*, cross section. (*F*) Amino acid comparison between *Xenopus* Fam3b, mouse FAM3B and human FAM3B. Mouse FAM3B and *Xenopus* Fam3b proteins share 85% and 63% identities to human FAM3B protein, as indicated. Mammalian FAM3B proteins have two signal peptide cleavage sites, indicated by arrowheads. Asterisks indicate conserved cysteine residues. In human FAM3B, Cys63-Cys91 and Cys69-Cys229 form two essential disulfide bridges. The multiple alignment of amino acid sequences was generated using ClustalOmega software and processed with BOXSHADE. The black and grey shaded boxes indicate identical and similar amino acids, respectively. Scale bars for (*C*), (*D-D"*) and (*E-E"*) all indicate 500 μm.

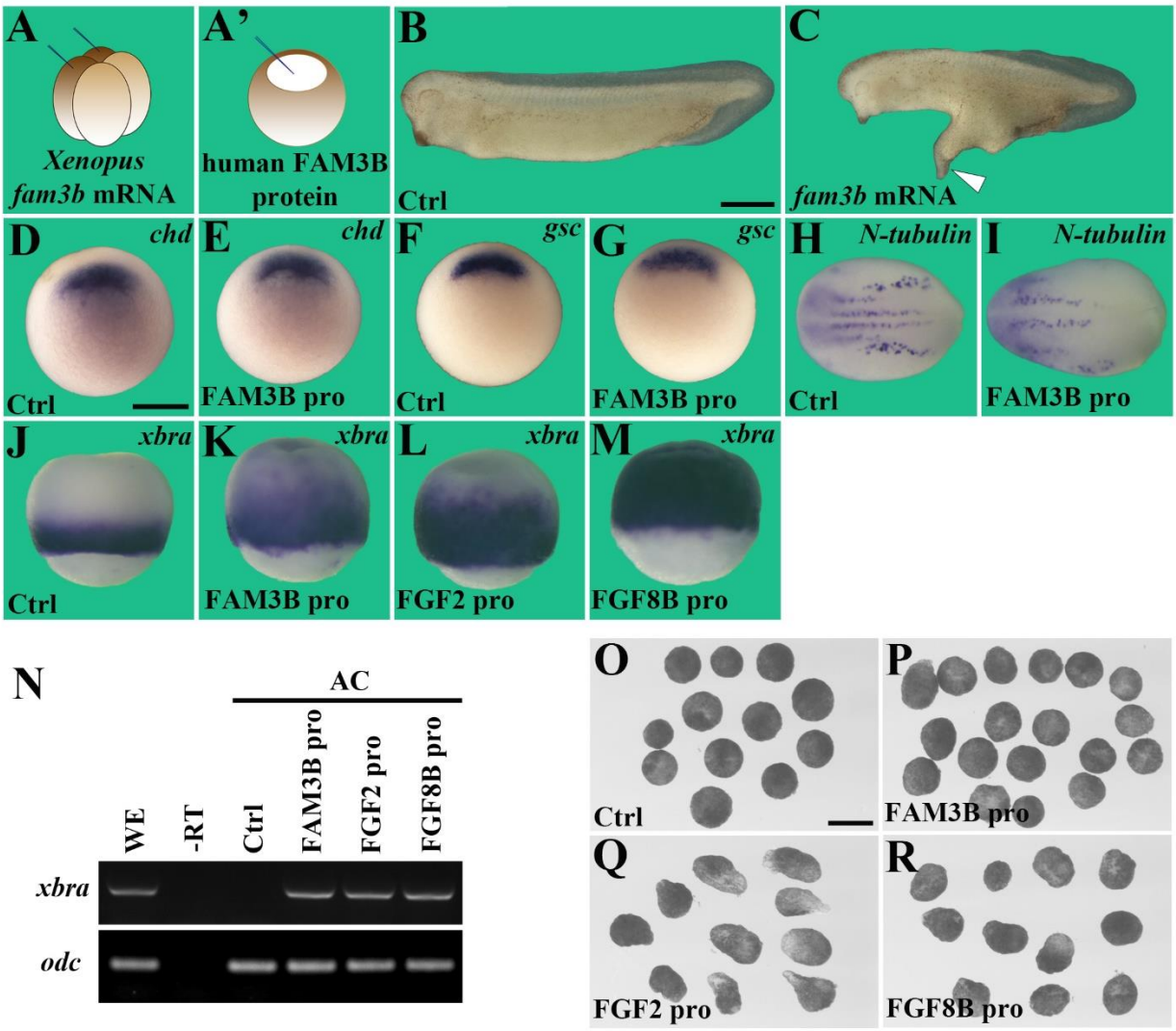


**Fig. S2.** Phenotype of *Xenopus fam3b* mRNA overexpression and effects of rhFAM3B protein microinjection on mesoderm formation and neural differentiation. (*A*) Schematic diagram depicting embryos injected with *Xenopus fam3b* mRNA into animal region of two ventral blastomeres at four-cell stage. (*A'*) Schematic diagram depicting injection of rhFAM3B protein into the blastocoele cavity of blastula stage embryos. (*B* and *C*) *Xenopus fam3b* mRNA injection leads to microcephaly and shortened A-P axis and induces tail-like structures. Four-cell stage embryos were injected with *Xenopus fam3b* mRNA (400 pg) into two ventral animal blastomeres and cultured until tailbud stage. Arrowhead indicates a secondary tail-like structure. Numbers of embryos analyzed were as follows: *B*, n=82, 100%; *C*, n=278, 37% with extra tails. (*D-I*) rhFAM3B protein has no effect on dorsal mesodermal markers *chd* and *gsc* and neural differentiation marker *N-tubulin*. BSA or rhFAM3B protein (40 nl at 15 μM) were injected into the blastocoele cavity of blastula stage embryos, which were cultured until stage 10.5 (*D-G*) or 16 (*H-I*) and subjected to WISH for *chd* (*D* and *E*), *gsc* (*F* and *G*) and *N-tubulin* (*H* and *I*). *D-G*, vegetal view with dorsal side up; *H* and *I*, dorsal view with anterior to the left. Unlike xFGF8, FAM3B does not induce ectopic neurons, suggesting it has some degree of receptor specificity. Numbers of embryos analyzed were as follows: *D*, n=37, 100%; *E*, n=45, 100%; *F*, n=32, 100%; *G*, n=38,

97%; *H*, n=24, 100%; *I*, n=27, 92%. (*J-M*) rhFAM3B protein, FGF2 protein and FGF8B protein strongly expand mesoderm marker *xbra*. BSA or rhFAM3B protein (40 nl at 15 μM, which amounts to 13.8 ng) or recombinant human FGF2 protein (1.25 ng) or recombinant human FGF8B protein (1.25 ng) were injected into the blastocoele cavity of blastula stage embryos, which were cultured until stage 11 and subjected to WISH with *xbra* and photographed in lateral view. Note that FAM3B is a potent mesoderm inducer. Numbers of embryos analyzed were as follows: *J*, n=31, 100%; *K*, n=36, 94%; *L*, n=41, 100%; *M*, n=37, 97%. (*N*) rhFAM3B protein, FGF2 protein and FGF8B protein all induce *xbra* expression in animal cap explants. Animal caps (AC) were excised from stage 8 embryos and cultured in the presence of BSA (Ctrl) or rhFAM3B protein (2 μg/ml) or recombinant human FGF2 protein (50 ng/ml) or recombinant human FGF8B protein (50 ng/ml) until stage 11 before isolating RNA to assess *xbra* expression by RT-PCR. *odc* was used as a loading control. -RT and whole embryo (WE) served as negative and positive controls, respectively. Data shown are representative of at least three independent experiments. (*O-R*) FGF2 protein and FGF8B protein, but not rhFAM3B protein, induce animal cap elongation. Animal caps excised and treated as in Fig. S2*N* were cultured until stage 16 and assessed for elongation. Note that FGF2 and FGF8B treated caps both elongated, although FGF8B to a lesser extent, whereas FAM3B protein-treated caps remained as round spheres. Data shown are representative of at least three independent experiments. Scale bars for (*B-C*), (*D-M*) and (*O-R*) all indicate 500 μm.

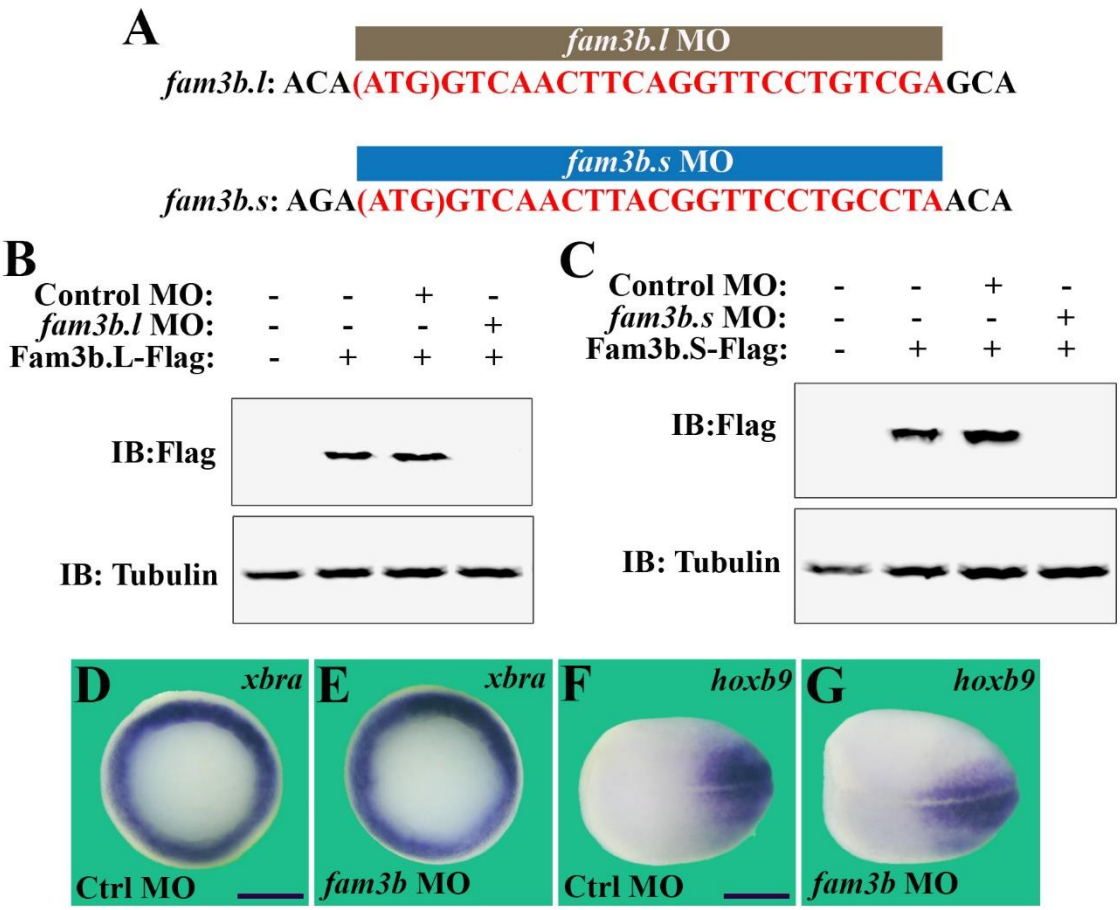


**Fig. S3.** Inhibition of *fam3b* mRNA translation by *fam3b* MO and effects of *fam3b* knockdown on mesoderm formation and posterior specification. (*A*) Illustration showing the target sites of *fam3b.l* and *fam3b.s* antisense MOs (red). The ATG start codon is shown in parenthesis. (*B* and *C*) *fam3b.l* and *fam3b.s* MOs inhibit the translation of *fam3b.l* and *fam3b.s* mRNAs, respectively. One-cell stage embryos were coinjected with *fam3b.l* or *fam3b.s* MO (20 ng each) and *fam3b.l* or *fam3b.s* mRNA (200 pg each), cultured until gastrula stage and subjected to immunoblotting with the indicated antibodies. α-Tubulin served as a loading control. (*D*-*G*) *fam3b* knockdown has no effect on expression of *xbra* and *hoxb9*. Embryos injected with control MO (120 ng) or *fam3b* MO (60 ng *fam3b.l* MO and 60 ng *fam3b.s* MO) at one-cell stage were cultured until stage 10.5 (*D* and *E*) or stage 16 (*F* and *G*) and processed for WISH with *xbra* (*D* and *E*) and *hoxb9* (*F* and *G*). The lack of inhibition of mesoderm and posterior development is attributed to redundant signaling factors. *D* and *E*, vegetal view; *F* and *G*, dorsal view with anterior to the left. Numbers of embryos analyzed were as follows: *D*, n=41, 100%; *E*, n=46, 91%; *F*, n=34, 100%; *G*, n=39, 84%. Scale bars for (*D* and *E*) and (*F* and *G*) both indicate 500 μm.

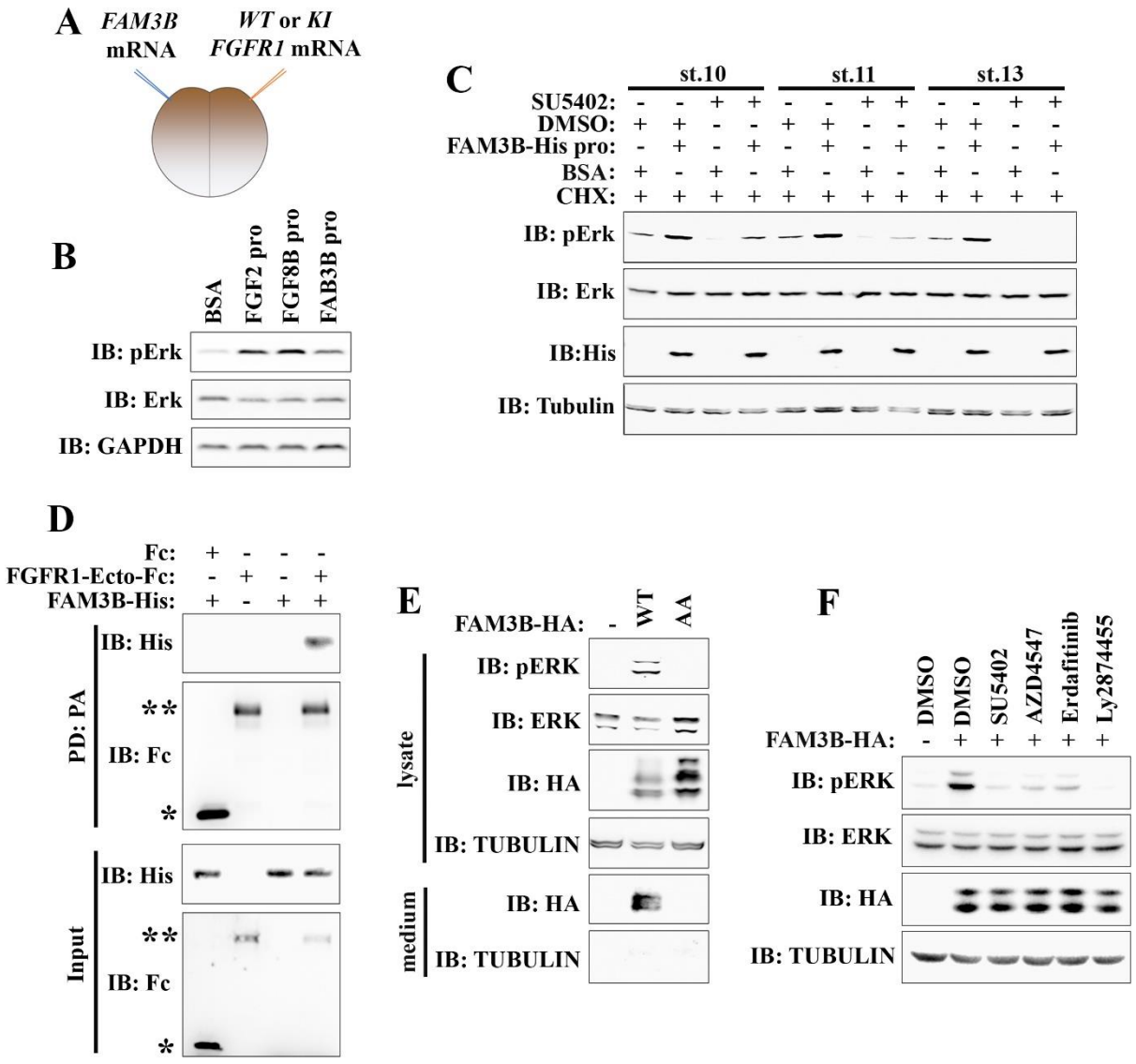


**Fig. S4.** FAM3B binds to FGFR1 ectodomain and activates ERK through FGFR. (*A*) Illustration showing two-cell stage embryos injected with *FAM3B* mRNA and wild type (WT) or kinase-inactive mutant (KI) *FGFR1* mRNA separately into different blastomeres. (*B*) rhFAM3B protein, FGF2 protein and FGF8B protein all activate ERK in *Xenopus* embryos. Blastula stage embryos injected with BSA or rhFAM3B protein (40 nl at 10 μM, which amounts to 9.2 ng) or recombinant human FGF2 protein (1.25 ng) or recombinant human FGF8B protein (1.25 ng) into the blastocoele cavity were cultured until stage 10 and then harvested for immunoblotting with indicated antibodies. GAPDH served as a loading control. (*C*) rhFAM3B protein activates ERK in an FGFR dependent and transcription independent manner at multiple stages in *Xenopus* embryos. Blastula stage embryos were injected with BSA or rhFAM3B protein (40 nl at 15 μM) into the blastocoele cavity and subsequently cultured in medium containing CHX (10 μg/ml) and DMSO or SU5402 (40 μM) until siblings reached gastrula and neurula stages. Embryos were then collected for immunoblotting with the indicated antibodies. α-Tubulin served as a loading control. Note that *Xenopus* has a single pErk band while mammalian cells have a pERK1/2 doublet. (*D*)

Specificity control showing that FAM3B binds to FGFR1-Ecto-Fc, but not Fc alone, *in vitro*. The experiment was performed as in Fig. 3*D* except for that Fc protein (500 ng) was used as a negative control and less FGFR1-Ecto-Fc protein (500 ng) was used. PD: pull-down, PA: Protein A magnetic beads. * indicates Fc and ** indicates FGFR1-Ecto-Fc. (*E*) Wild type, but not a mutant form of FAM3B lacking disulfide bonds activates ERK. HEK293T cells were transfected with wild type or mutant FAM3B (C91A/C229A). 20 hours after transfection, cells were serum starved for 24 hours and both lysate and medium were collected for immunoblotting with indicated antibodies. α-Tubulin served as a loading control. AA, FAM3B C91A/C229A mutant. (*F*) ERK activation induced by FAM3B DNA transfection into HEK293T cells requires FGFR activity. 20 hours after transfection with human FAM3B DNA, cells were serum starved for 24 hours and treated with the FGFR inhibitors SU5402 (20 μM), AZD4547 (1 μM), Erdafitinib (1 μM) or Ly2874455 (1 μM) for 3 hours. Finally, cells were harvested for immunoblotting with the indicated antibodies. α-Tubulin served as loading control.

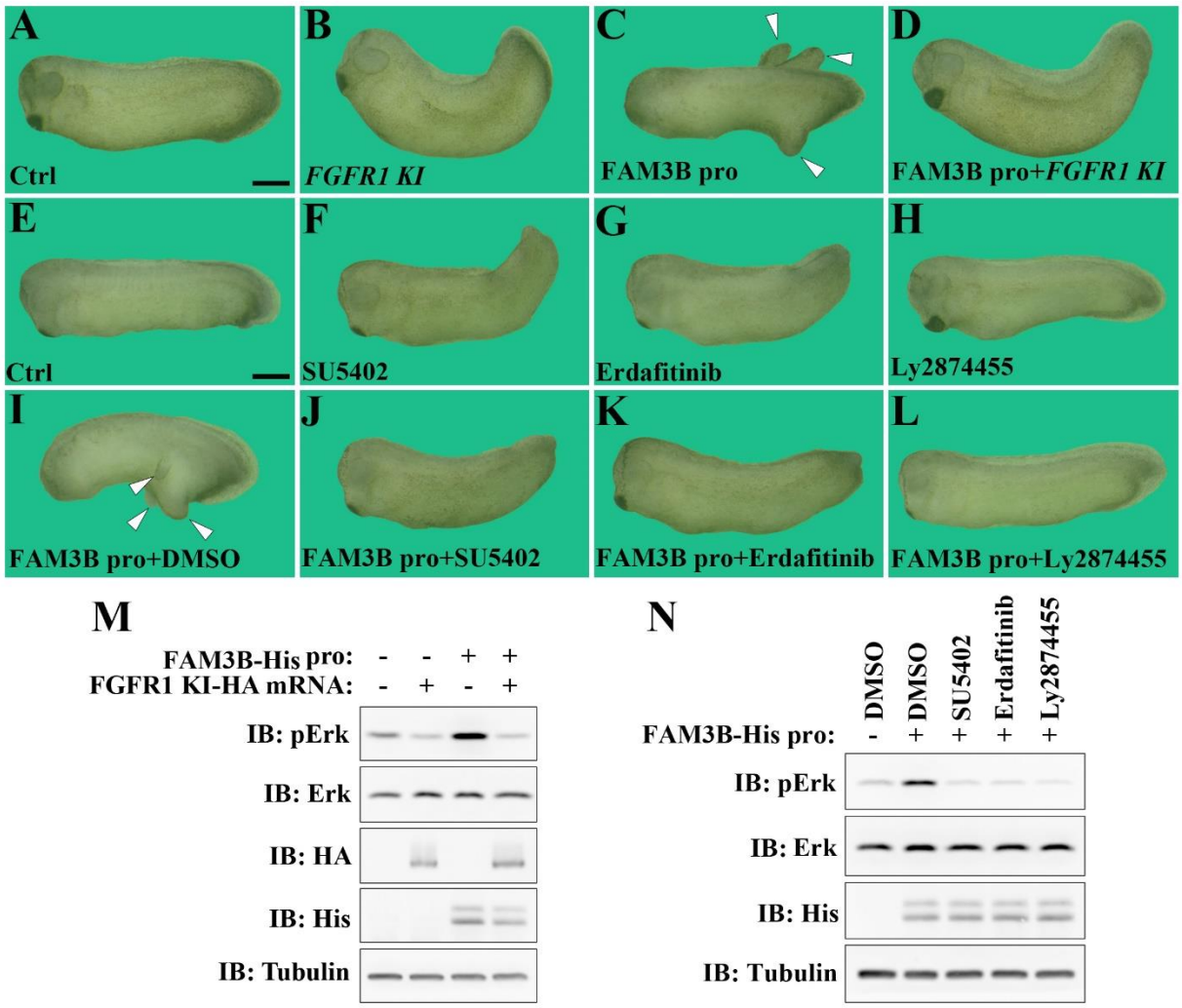


**Fig. S5.** FGFR inhibition blocks rhFAM3B protein-induced posterior abnormalities and ERK activation in *Xenopus* embryos. (*A-D*) FGFR1 KI overexpression blocks rhFAM3B protein-induced microcephaly and formation of ectopic tail-like structures. Embryos were first injected with *FGFR1 KI* mRNA (100 pg) at one-cell stage and then injected with rhFAM3B protein (40 nl at 15 μM) into the blastocoele cavity at blastula stage as indicated. Phenotypes were analyzed at tailbud stage. Arrowheads in (*C*) indicate ectopic tail-like structures. Scale bar: 500 μm. Numbers of embryos analyzed were as follows: *A*, n=43, 100%; *B*, n=38, 94% with enlarged heads and defective trunks); *C*, n=56, 96%; *D*, n=51, 90%. (*E-L*) FGFR inhibitors block rhFAM3B protein-induced microcephaly and formation of ectopic tail-like structures *in vivo*. Blastula stage embryos were injected with rhFAM3B protein (40 nl at 15 μM) into the blastocoele cavity and then treated with SU5402 (20 μM), Erdafitinib (2 μM), or Ly2874455 (20 nM). Phenotypes were analyzed at tailbud stage. Arrowheads in (*I*) indicate ectopic tail-like structures. Scale bar: 500 μm. Numbers of embryos analyzed were as follows: *E*, n=36, 100%; *F*, n=41, 92%; *G*, n=45, 91%; *H*, n=40, 87%; *I*, n=46, 97%; *J*, n=52, 86%; *K*, n=47, 82%; *L*, n=49, 87%. (*M* and *N*) FGFR1 KI overexpression or FGFR inhibitors blocked rhFAM3B protein-induced ERK activation in *Xenopus* embryos. Embryos were microinjected, or not, at two-cell stage with 400 pg of *FGFR1 KI* mRNA and at blastula stage with rhFAM3B protein (40 nL at 15 μM), lysed at gastrula stage and subjected

to immunoblotting with the indicated antibodies. α-Tubulin served as a loading control. Note that ERK activation by FAM3B in *Xenopus* embryos requires FGFR activity.

## SI References


1. M. Phelps, P. Stuelsatz, Z. Yablonka-Reuveni, Expression profile and overexpression outcome indicate a role for beta Klotho in skeletal muscle fibro/adipogenesis. *Febs J* **283**, 1653-1668 (2016).
2. G. Colozza, E. M. De Robertis, Maternal syntabulin is required for dorsal axis formation and is a germ plasm component in Xenopus. *Differentiation* **88**, 17-26 (2014).
3. R. David, D. Wedlich, PCR-based RNA probes: a quick and sensitive method to improve whole mount embryo in situ hybridizations. *Biotechniques* **30**, 769-772, 774 (2001).