SUPPLEMENTARY MATERIAL

Type-IV Antifreeze Proteins are Essential for Epiboly and Convergence in Gastrulation of Zebrafish Embryos

Qing Xiao[1], Jian-Hong Xia[1], Xiao-Juan Zhang, Zhi Li, Yang Wang, Li Zhou, Jian-Fang Gui*

State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, University of the Chinese Academy of Sciences, Wuhan 430072, China

[1] These authors contributed equally to this work.

*Corresponding author: Tel: +86-27-68780707; Fax: +86-27-68780123; E-mail: jfgui@ihb.ac.cn

Supplementary figure legends

Fig. S1. Schematic diagram of genomic organization of *afp4a* and *afp4b* in gibel carp and zebrafish. Arrows above the boxes (exons) indicate translation initiation sites. The thin lines indicate introns. The red and blue boxes indicate ORFs of *afp4a* and *afp4b*, respectively; and the white boxes indicate non-coding sequences. The numbers under the lines indicate the nucleotide numbers of each intron; the numbers above the boxes indicate the nucleotide numbers of each exon.

Fig. S2. The longitudinal sections of embryos stained by *afp4a* and *afp4b* antisense probes. Shield (A, D), 75% epiboly (B, E) and bud (C, F) stage *in situ* hybridized embryos for *afp4a*/*afp4b* (A-C) or *afp4b* (D-F). Dorsal to the right. Red arrow indicates YSL; black arrow indicates yolk; black arrowhead indicates blastoderm.

Fig. S3. Tests of the validity and specificity of MOs. (A) Tests of the translation blocking by co-injection with *afp4a:EGFP* or *afp4b:EGFP* mRNA. Left pictures show representative images of EGFP expression in bud stage embryos injected with *afp4a:EGFP* (100 pg/embryo), *afp4b:EGFP* (100 pg/embryo), *afp4a:EGFP* plus *afp4a*-tb-MO (8 ng/embryo), and *afp4b:EGFP* plus *afp4b*-tb-MO (2.5 ng/embryo). Right histogram displays the quantitative analysis results of the inhibition of *afp4a:EGFP* or *afp4b:EGFP* expression by co-injection *afp4a*-tb-MO (columns indicate by a red box on the left) or *afp4b*-tb-MO (columns indicate by a blue box on the right). Results are presented as means ± SD of three independent experiments. Alphabetic letters illustrate group differences based on post hoc test (SNK) following ANOVA ($P < 0.01$). (B) Tests of the splice change by semi-quantitative RT-PCR. The end products are shown schematically and by RT-PCR analyses after *afp4a*-sb-MO (left) or *afp4b*-sb-MO (right) injection. In schematic diagrams, the thin lines indicate introns. The red or blue boxes indicate ORFs of *afp4a* or *afp4b*, respectively; and the white boxes indicate non-coding sequences. Black arrows indicate translation initiation sites, green arrows indicate the position of primers applied in RT-PCR, and the nucleotide numbers of RT-PCR product are shown in green. Black numbers under the diagrams and in boxes show the corresponding nucleotide numbers. Purple lines above the boxes indicate the locations of sb-MOs, which is targeted to the intron 3/exon 4 boundary. Alternative splicing introduced early stop codons (TAA) are indicated by pink letters shortly after the exon 3/intron 3 junction, and the nucleotide numbers of WT ORF in final products are displayed above diagram in red (*afp4a*) or blue (*afp4b*) numbers. Analysis of defective splicing in *afp4a*-sb-MO (4 ng/embryo) morphants at shield stage is shown at left. Amplification of a bigger RT-PCR fragment (426 bp) than that from WT embryos (320 bp) was observed with primers for *afp4a*. Analysis of changed splicing in *afp4b*-sb-MO (8 ng/embryo) morphants at bud stage is shown at right. Lesser 323 bp RT-PCR products than those from WT embryos were observed, and a larger band (2584 bp) was detected with primers for *afp4b*. (C) Representative images of bud stage embryos injected with Ctrl-MO (8 ng/embryo), *afp4a*-tb-MO (8 ng/embryo),

afp4a-sb-MO (4 ng/embryo), *afp4b*-tb-MO (2.5 ng/embryo) or *afp4b*-sb-MO (8 ng/embryo). Lateral views, animal pole is up and dorsal is to the right.

Fig. S4. Whole-mount *in situ* hybridization detection of germ layer differentiation and CE movement in *afp4a*-sb-MO morphants. In each section, expression of marker genes in *afp4a*-sb-miMO embryos (left) and *afp4a*-sb-MO morphants (right) are shown. (A) *foxA3*, shield stage. (B) *gsc*, shield stage. (C) *ntl*, shield stage. (D) *ntl*, bud stage. (E) *myoD*, bud stage. (F) *myoD*, 8-somite stage. (A-F) Dorsal views, animal pole to the top. (G) *ntl*, bud stage, lateral view, dorsal to the right, arrows indicate the anterior boundary of *ntl* expression. (H) *hgg1* and *dlx3b*, bud stage, top views, ventral up. (I) *pax2.1*, 8-somite stage, lateral views, dorsal to the right. os, optic stalk; m, the midbrain hindbrain boundary; o, otic vesicles; p, pronephros.

Supplementary table legends

Table S1. Comparison of exon/intron boundaries between *Cagafp4*s and *Drafp4*s. Exon sequence is in upper case and intron sequence is in lower case. Differential nucleotides are indicated by grey background.

Table S2. Comparison of exon/intron boundary sequences between *Drafp4a* and *Drafp4b*. Exon sequence is in upper case and intron sequence is in lower case. Differential nucleotides are indicated by grey background. Targets of sb-MOs are boxed in black, and the premature stop codons are boxed in grey.

Supplementary figures

Fig. S1

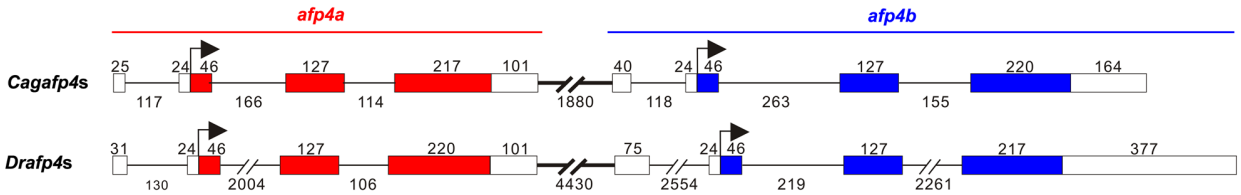


Fig. S2

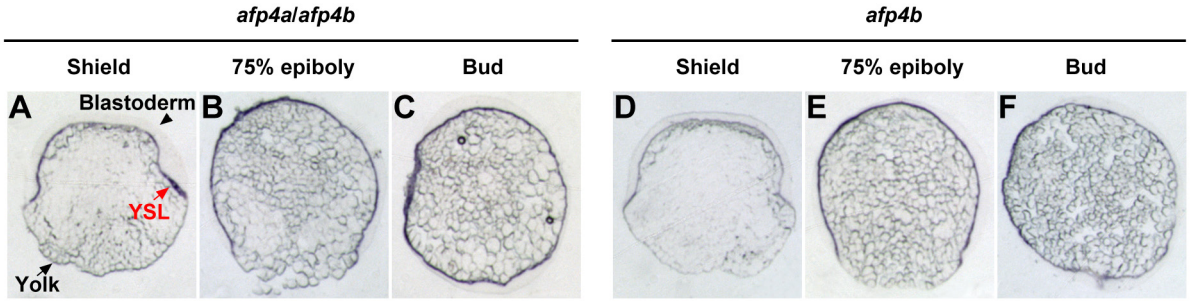
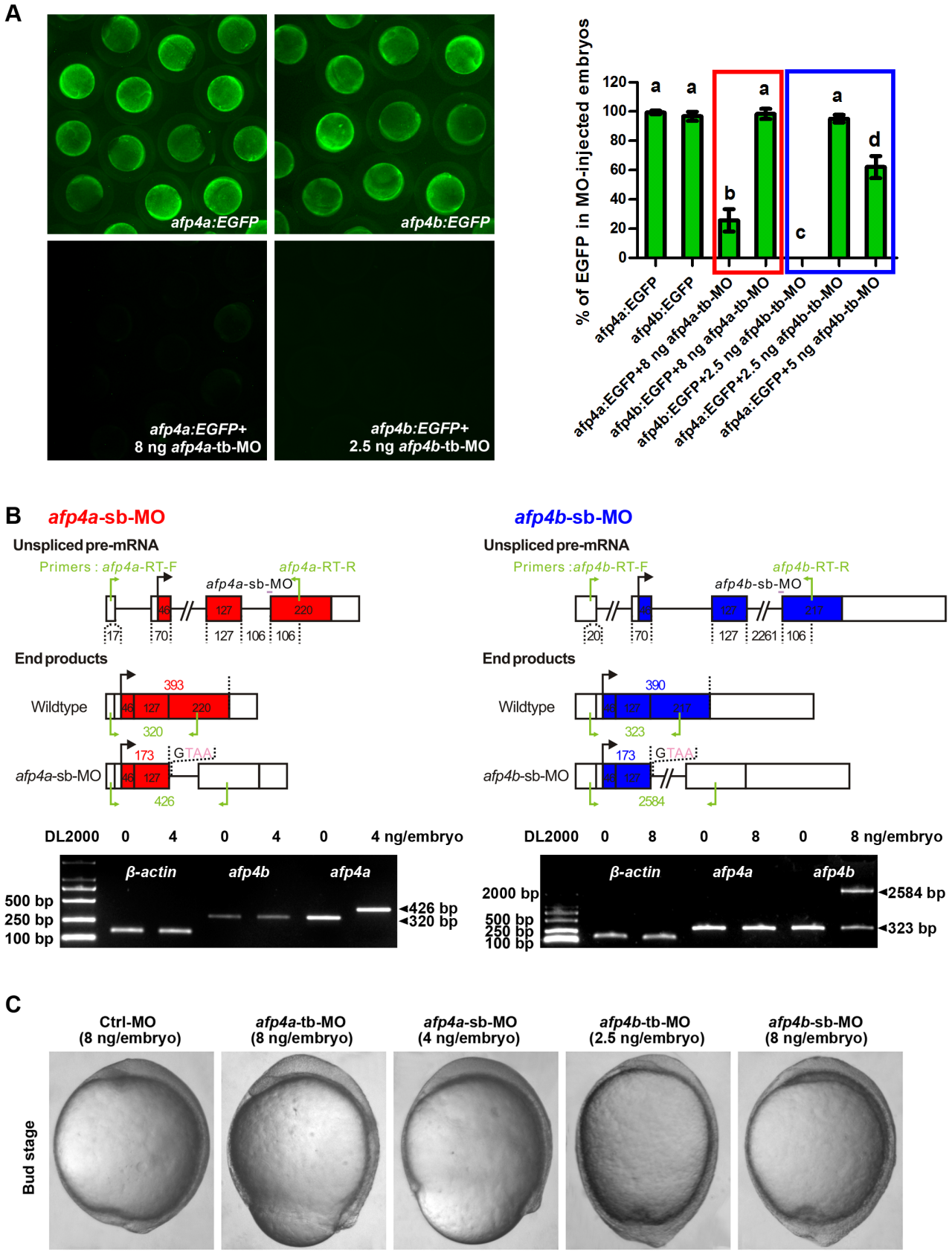


Fig. S3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afp4a:EGFP
afp4b:EGFP
afp4a:EGFP+
8 ng afp4a-tb-MO
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2.5 ng afp4b-tb-MO
% of EGFP in MO-injected embryos
120
100
80
60
40
20
0
a
a
b
a
c
a
d
afp4a:EGFP
afp4b:EGFP
afp4a:EGFP+8 ng afp4a-tb-MO
afp4b:EGFP+8 ng afp4a-tb-MO
afp4b:EGFP+2.5 ng afp4b-tb-MO
afp4a:EGFP+2.5 ng afp4b-tb-MO
afp4a:EGFP+5 ng afp4b-tb-MO
B
afp4a-sb-MO
Unspliced pre-mRNA
Primers : afp4a-RT-F
afp4a-RT-R
afp4a-sb-MO
17
70
127
106
106
End products
Wildtype
393
320
afp4a-sb-MO
173
GTAA
426
DL2000 0 4 0 4 0 4 ng/embryo
β-actin
afp4b
afp4a
500 bp
250 bp
100 bp
426 bp
320 bp
afp4b-sb-MO
Unspliced pre-mRNA
Primers :afp4b-RT-F
afp4b-RT-R
afp4b-sb-MO
20
70
127
2261
106
End products
Wildtype
390
323
afp4b-sb-MO
173
GTAA
2584
DL2000 0 8 0 8 0 8 ng/embryo
β-actin
afp4a
afp4b
2000 bp
500 bp
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2584 bp
323 bp
C
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(8 ng/embryo)
afp4a-tb-MO
(8 ng/embryo)
afp4a-sb-MO
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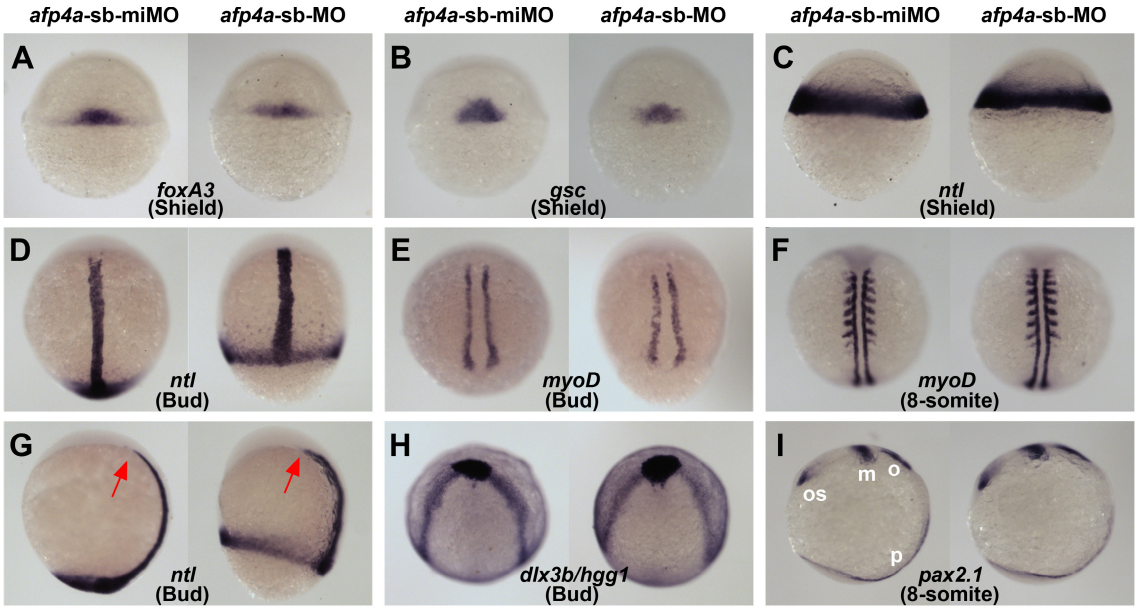
(8 ng/embryo)
Bud stage

Fig. S4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A
foxA3
(Shield)
B
gsc
(Shield)
C
ntl
(Shield)
D
ntl
(Bud)
E
myoD
(Bud)
F
myoD
(8-somite)
G
ntl
(Bud)
H
dlx3b/hgg1
(Bud)
I
m
o
os
p
pax2.1
(8-somite)

Supplementary tables

Table S1. Comparison of exon/intron boundaries between *Cagafp4*s and *Drafp4*s

Gene	Species	NO. of exon/intron boundary	Sequence	NO. of intron/exon boundary
afp4a	*Cag*	Exon 1	AGGTCTAAGAACTCCTGTAAGCAAGgtgcaattatatctacattatttgt	
	Dr	/Intron 1	AGGTCTAACAATCTCTGGACGCAAGgtaagattacagctatgttaaacct	
	Cag		ttcattattgtctctttctgttcagGCAGCCCTCACCCAACCTACAACAA	Intron 1
	Dr		gaaatgattgtttttgtgtttcagGCAACTCTTGCTCAACCCGCAATCA	/Exon 2
	Cag	Exon 2	GTCCTTGTTACTGCTCTGGCCATTGgtgagactttaaaccatgttaactt	
	Dr	/Intron 2	GTCATTGTTGTTGCTCTGGCCATCGgtgagtctttaaaccacattttgtc	
	Cag		tcaaacatctctgctcgtattccagGCTCTGAATCAGTTTCTCTGGTCAA	Intron 2
	Dr		tcaaacatttctgttcatgtt-cagGCTCTGAGTCAGCATCTCTGGTCAA	/Exon 3
	Cag	Exon 3	GCCCAGAGCTGGTCAACAAGGCCAAgtaagtccatcatcatcatcatcca	
	Dr	/Intron 3	GTGCTGAGCTGGCCAACAAGGCCAAgtaagtccatcatctcaactttgaa	
	Cag		gagttctctttggtttgtgttgcagCGCTTACTTTGAGCAGAGCAGAGCC	Intron 3
	Dr		ttgttttttgtttgttcatggcacagTGCTTACCTGGAGCAGAGCAGAGCT	/Exon 4
afp4b	*Cag*	Exon 1	AGGTCTAAAAACCCCTGGATACAAGgtaagattacagctacc-ttatgtg	
	Dr	/Intron 1	GCCTATATCGTCACATTGACACAAGgtaaggacacagctaatattatgtg	
	Cag		tgaactattgtctctttctgttcagGCAACTCTTACCCAACCTGCAACAA	Intron 1
	Dr		ttaatgattgtctttgtgtttcagGCAACCCTGGCTCATCCCACAATCA	/Exon 2
	Cag	Exon 2	ATCCTTGTTGTTGCTCTGGCCATTGgtgagactttaaatcatgttaactt	
	Dr	/Intron 2	ATCCTTGTTGTTGCTCTGGCCATCCgtgagtctttaaaccatgttttgtc	
	Cag		tcaaacatctctgctcgtattccagGCTCTGAATCAGTATCTCTGGTCAA	Intron 2
	Dr		tcaaacatttctgttcatgtt-cagGCTCTGAGTCAGCATCTCTGGTCAA	/Exon 3
	Cag	Exon 3	GCCCTGAGCTGGTCAACAAGGCCAAgtaagtccatgagc-ttcgtcttca	
	Dr	/Intron 3	GTCCAGAGCTGGCCAACAAAGCCAAgtaagtccatcatcattcatcatca	
	Cag		tgattatctttggtttgtgttgcagCGCTTACTTTGAGCAGAGCAGAGCC	Intron 3
	Dr		tctgcgtattgtctttgtgatgcagTGCTTACCTGGAGGAGAGCAGAGCC	/Exon 4

Table S2. Comparison of exon/intron boundary sequences between *Drafp4a* and *Drafp4b*

Gene	NO. of exon/intron boundary	Sequence	NO. of intron/exon boundary
afp4a	Exon 1/Intron 1	AGGTCTAACAATCTCTGGACGCAAGgtaagattacagcta-tgttaaacc	
afp4b		GCCTATATCGTCACATTGACACAAGgtaaggacacagctaatattatgtg	
afp4a		gaaatgattgtttttgtgttttcagGCAACTCTTGCTCAACCCGCAATCA	Intron 1/Exon 2
afp4b		ttaatgattgtctttgtgttttcagGCAACCCTGGCTCATCCCACAATCA	
afp4a	Exon 2/Intron 2	GTCATTGTTGTTGCTCTGGCCATCGgtgagtctttaaaccacattttgtc	
afp4b		ATCCTTGTTGTTGCTCTGGCCATCCgtgagtctttaaaccatgttttgtc	
afp4a		atcaaacatttctgttcatgttcagGCTCTGAGTCAGCATCTCTGGTCAA	Intron 2/Exon 3
afp4b		atcaaacatttctgttcatgttcagGCTCTGAGTCAGCATCTCTGGTCAA	
		early stop codon	
afp4a	Exon 3/Intron 3	GTGCTGAGCTGGCCAACAAGGCCAAgtaagtccatcatctcaactttgaa	
afp4b		GTCCAGAGCTGGCCAACAAAGCCAAgtaagtccatcatcattcatcatca	
		sb-MO	
afp4a		ttgtttttgtttgttcatggcacagTGCTTACCTGGAGCAGAGCAGAGCT	Intron 3/Exon 4
afp4b		tctgcgtattgtctttgtgatgcagTGCTTACCTGGAGGAGAGCAGAGCC	