



**HAL**  
open science

## Tissue-Specific Alternative Splicing of Tak1 Is Conserved in Deuterostomes.

Julian P Venables, Emmanuel Vignal, Stephen Baghdiguian, Philippe Fort,  
Jamal Tazi

► **To cite this version:**

Julian P Venables, Emmanuel Vignal, Stephen Baghdiguian, Philippe Fort, Jamal Tazi. Tissue-Specific Alternative Splicing of Tak1 Is Conserved in Deuterostomes.. *Molecular Biology and Evolution*, 2012, 29 (1), pp.261-269. 10.1093/molbev/msr193 . hal-00649579

**HAL Id: hal-00649579**

**<https://hal.science/hal-00649579v1>**

Submitted on 15 Jun 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License

# Tissue-Specific Alternative Splicing of Tak1 Is Conserved in Deuterostomes

Julian P. Venables,<sup>1</sup> Emmanuel Vignal,<sup>2</sup> Stephen Baghdiguian,<sup>3</sup> Philippe Fort,<sup>\*2</sup> and Jamal Tazi<sup>\*,1</sup>

<sup>1</sup>Institut de Génétique Moléculaire de Montpellier, UMR 5535, CNRS, Université Montpellier 2, Montpellier, France

<sup>2</sup>Centre de Recherche de Biochimie Macromoléculaire, UMR 5237, CNRS, Universités Montpellier 2 et 1, Montpellier, France

<sup>3</sup>Institut des Sciences de l'Evolution, UMR 5554, CNRS, Université Montpellier 2, Montpellier, France

**\*Corresponding authors:** E-mail: jamal.tazi@igmm.cnrs.fr; philippe.fort@crbm.cnrs.fr.

**Associate editor:** William Jeffery

## Abstract

Alternative splicing allows organisms to rapidly modulate protein functions to physiological changes and therefore represents a highly versatile adaptive process. We investigated the conservation of the evolutionary history of the “Fox” family of RNA-binding splicing factors (RBFox) as well as the conservation of regulated alternative splicing of the genes they control. We found that the RBFox proteins are conserved in all metazoans examined. In humans, Fox proteins control muscle-specific alternative splicing of many genes but despite the conservation of splicing factors, conservation of regulation of alternative splicing has never been demonstrated between man and nonvertebrate species. Therefore, we studied 40 known Fox-regulated human exons and found that 22 had a tissue-specific splicing pattern in muscle and heart. Of these, 11 were spliced in the same tissue-specific manner in mouse tissues and 4 were tissue-specifically spliced in muscle and heart of the frog *Xenopus laevis*. The inclusion of two of these alternative exons was also downregulated during tadpole development. Of the 40 in the starting set, the most conserved alternative splicing event was in the transforming growth factor (TGF) beta-activated kinase Tak1 (MAP3K7) as this was also muscle specific in urochordates and in Ambulacraria, the most ancient deuterostome clade. We found exclusion of the muscle-specific exon of Tak1 was itself under control of TGF beta in cell culture and consistently that TGF beta caused an upregulation of Fox2 (RBFox2) expression. The alternative exon, which codes for an in-frame 27 amino acids between the kinase and known regulatory domain of TAK1, contains conserved features in all organisms including potential phosphorylation sites and likely has an important conserved function in TGF beta signaling and development. This study establishes that deuterostomes share a remarkable conserved physiological process that involves a splicing factor and expression of tissue-specific isoforms of a target gene that expedites a highly conserved signaling pathway.

**Key words:** alternative splicing, evolution, tissue specificity, TGF beta signaling.

## Introduction

Alternative splicing enables a limited number of genes to encode a far greater number of proteins in all multicellular organisms (Black 2003; Elliott and Ladomery 2011). Alternative isoforms can be expressed in a tissue-specific and temporally regulated manner under the control of pre-mRNA-binding proteins. For example, the neural-specific splicing factor Nova has been shown to control neural-specific splicing of five (*aplp2*, *brd9*, *kcnma1*, *neo1*, and *ptprf*) conserved target exons in mammals and fish (Jelen et al. 2007). A homolog of the Nova protein (*pasilla*) has been found in flies, and genome-wide studies have identified common target genes in flies and mammals, although these were not found to be in orthologous exons (Brooks et al. 2011). Indeed, regulated alternative exons that are conserved between deuterostome and protostome species are likely to be extremely rare, as the two lineages have undergone extreme adaptations in their splicing programs. Consistent with this expectation, Nova is expressed in neurons in vertebrates but predominantly in the salivary gland of protostomes (Irimia et al. 2011).

The original Fox (feminizing on the X) protein was discovered in *Caenorhabditis elegans*, and there are three

homologs in mammals, RBFox1, RBFox2, and RBFox3 (Kuroyanagi 2009). The proteins are known to function as controllers of alternative splicing by binding to UGCAUG sequences in RNA. Furthermore, they have an exquisite mode of action whereby binding downstream of exons enhances their recognition and binding upstream within the extended 3' splice site, branch point, and polypyrimidine tract inhibits exon inclusion. Fox proteins control alternate splicing of many different genes in heart and muscle in mammals (Das et al. 2007; Kalsotra et al. 2008; Bland et al. 2010; Han et al. 2011) among which are several involved in cytoskeletal remodeling and invasion (Das et al. 2007; Zhang et al. 2008; Venables et al. 2009; Yeo et al. 2009). Fox proteins also likely control inclusion of alternative exons in their own genes, allowing them to autoregulate their own activity (Damianov and Black 2010).

Despite the conservation of splicing factors between *C. elegans* and man, conservation of tissue-specific alternative splicing between vertebrates and other taxa had not been described. To address this issue, we looked in diverse organisms for the presence of Fox genes and found them to

be conserved in all metazoans. We also looked at the alternative splicing pattern of 40 established targets of the human splicing factor Fox2, including the transforming growth factor (TGF) beta-activated kinase TAK1/MAP3K7 (Venables et al. 2009). TAK1 is the major intracellular mediator of the highly conserved TGF beta/BMP signaling pathway (Shibuya et al. 1998; Yamaguchi et al. 1999; Massague 2008) and it is implicated in many other different signaling pathways including TNF and interleukin as well as affecting JNK and p38 activities (Li et al. 2003). TAK1 was also recently shown to be essential for muscle development and the differentiation-associated activation of p38 and Akt kinases (Bhatnagar et al. 2010). Tak1 is expressed as four tissue-specific isoforms (Kondo et al. 1998; Dempsey et al. 2000). The two major isoforms differ by a 27 amino acid stretch encoded by the short exon that we previously identified as a putative Fox2 target but no difference in function has been demonstrated between the two isoforms, and the longer isoform has scarcely been considered before.

Here, we show that muscle-specific exclusion of an alternative exon of Tak1 is conserved in all deuterostomes examined. Gene comparison in organisms from mammals to sea urchin and acorn worm reveals an exquisite use of alternative splicing strategies during evolution. Although little attention has been hitherto given to TAK1 isoforms, our study shows that the Tak1 gene has the most conserved regulated alternative splicing pattern so far reported, which supports basic and distinct functional roles for TAK1 isoforms in TGF beta signaling.

## Materials and Methods

### Reverse Transcriptase-Polymerase Chain Reaction

Cells were harvested in Tri reagent (Sigma) and, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with first strand cDNA synthesis kit from GE HealthCare. RT-PCR reactions from 60-ng RNA were used as template for a 50- $\mu$ l PCR reaction with 50 pmol of each primer (listed in [supplementary table S1, Supplementary Material](#) online) using platinum Taq (Invitrogen) at 94 °C for 2 min then 35 cycles of 94 °C, 55 °C and 72 °C for 30 s each then 2 min at 72 °C. PCR products were run on a 1.5% agarose TBE gel and stained with ethidium bromide for imaging. For sea urchin whose Tak1 gene shows high G + C content, reverse transcription was performed with the maxima first strand cDNA synthesis kit (Fermentas) and included a 0.5  $\mu$ M specific primer GAGCCATCCTC ATGTGTTCA. Ten rounds of nested PCR were then performed with forward and reverse primers TTCAGTGATCT TCCCCCTGT and TGGAATTAGGATTGGGTGGA, and 0.5  $\mu$ l was used in a hot start for 35 rounds with forward and reverse primers CCGACCTCAAAGGAACAGAA and ACCACCTCTGCTCCAGTGAC.

Quantification of PCR ratios was performed with GeneTools software (Syngene) using automatic band assignment and background correction. Raw band volumes were converted to concentrations in arbitrary units by dividing by the PCR product size, and percent spliced in values were cal-

culated by dividing the long band concentration by the sum of the long and short (see [supplementary table S1, Supplementary Material](#) online).

### Database Searches

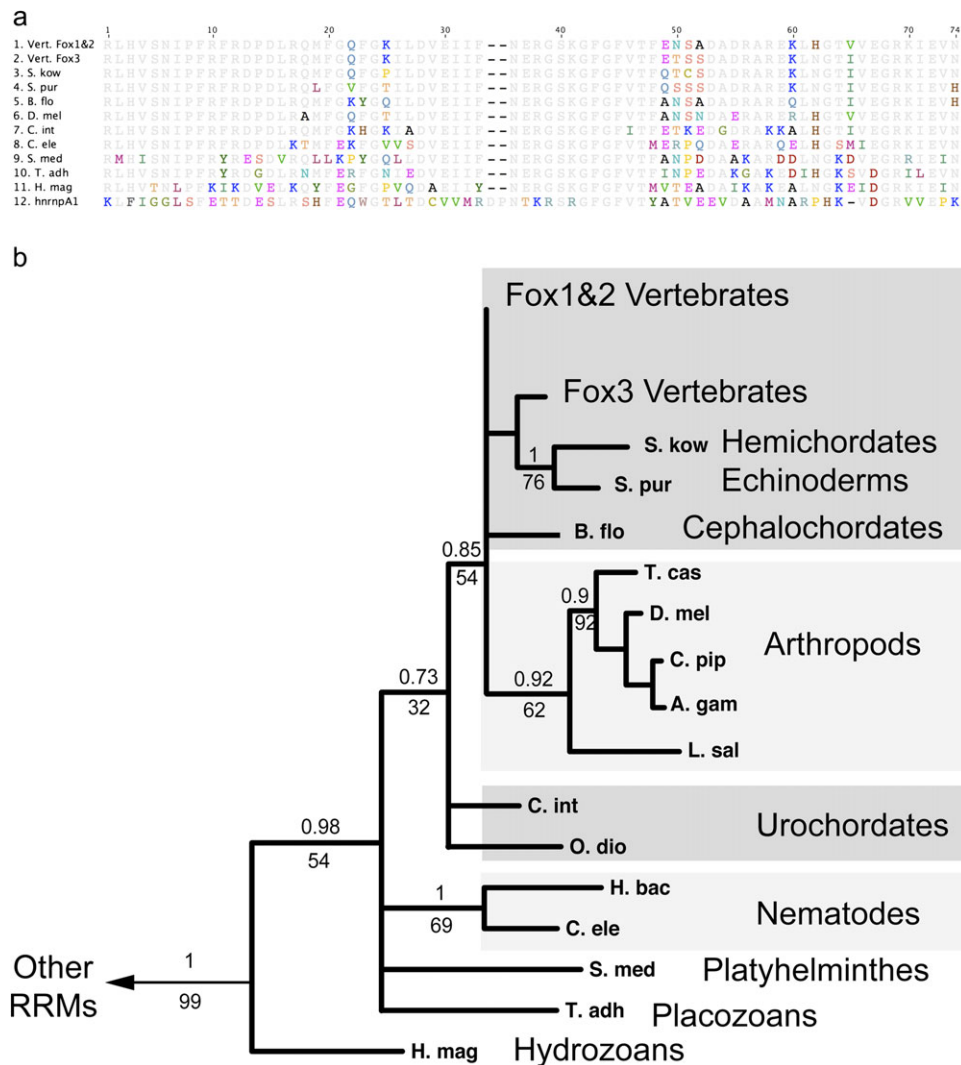
Blastp and tblastn searches for RBFox, Tak1/MAP3K7, Sec3/ExoC1, MYO18A, and SYNE2 orthologous sequences in nr, reference genomic sequences, reference mRNA, and EST databases were performed using the NCBI Blast suite <http://blast.ncbi.nlm.nih.gov/>. Short alternative exons were identified by Staden diagen analysis of introns translated into three reading frames.

### Phylogenetic Inference

Trees were inferred by using MrBayes (Huelsenbeck and Ronquist 2001) and PhyML (Guindon et al. 2010). Kinase (Tak1 kinase) or RRM (Fox splicing factors) sequences ([supplementary tables S2 and S3, Supplementary Material](#) online) were aligned with MAFFT (Katoh et al. 2002). Alignments were analyzed with ProtTest (v. 10.2) to identify the best substitution models (Abascal et al. 2005). We used MrBayes 3.1.2 with the wag matrix rate and a gamma distribution describing among-site rate variation with eight categories (+G8). MCMCMC chains were run for 1 million generations with a sample frequency of 1,000 and a 10% burn in value. For maximum likelihood (ML) analyses, we also used the wag + G8 in PhyMLM while searching for the ML tree by performing both nearest neighbor interchange and subtree pruning and regrafting topological moves on a bioNJ starting tree. The statistical robustness of inferred nodes was assessed by 100 bootstrap pseudoreplicates of the same ML search. Whatever the method and the data set (amino acids or codons) trees inferred showed the same topologies. Silent versus nonsilent  $K_s/K_a$  ratios of substitutions between *Ciona intestinalis* and *Ciona savignyi* Fox and Tak1 open reading frames ([supplementary table S4, Supplementary Material](#) online) were calculated using the KaKs calculator program (Zhang et al. 2006).

### Cell Culture

Normal murine mammary gland (NMuMG) cells were cultivated at 37 °C in the presence of 5% CO<sub>2</sub> in Dulbecco's modified Eagle medium supplemented with 10% (v/v) fetal calf serum. Cells were plated in 12-mm glass cover slips 16–24 h before TGF beta stimulation. After TGF beta stimulation (5 ng.ml<sup>-1</sup>), cells were fixed for 10 min in 3.7% (v/v) formalin-phosphate-buffered saline (PBS). After 10 min permeabilization in 0.1% Triton X-100 in PBS and a 30 min incubation at room temperature in 0.1% bovine serum albumin (BSA)-PBS, cells were processed for immunohistochemistry. Cover slips were incubated for 1 h at 37 °C with the anti-E-cadherin antibody (0.25  $\mu$ g.ml<sup>-1</sup> in PBS 0.1% BSA, BD Transduction Laboratories), washed twice with PBS, then stained for F-actin using rhodamine-conjugated phalloidin. Cells were washed with PBS, mounted in Mowiol (Aldrich) and visualized by fluorescence microscopy (AX10 Imager.M1, Zeiss). Images were captured with an AxioCam MRm camera (Zeiss). Western blotting was performed with



**FIG. 1.** Evolution of RNA-binding Fox proteins in fungi/metazoa. (a) Amino acid sequences of RRM domains identified in genome and EST databases were aligned. 1. In vertebrates, RRM domains are identical for RBFox1 and RBFox2 (Vert. Fox1 and 2); 2. RBFox3 (Vert. Fox3); 3. *S. kow*: Acorn worm; 4. *S. pur*: Sea urchin; 5. *B. flo*: Lancelet; 6. *Drosophila melanogaster*: Fruit fly; 7. *Ciona intestinalis*: Sea squirt; 8. *C. elegans*: Nematode Worm; 9. *S. med*: Planarian; 10. *T. adh*: Placozoan; 11. *H. mag.*: Jellyfish; 12. The RRM domain of *hnrnpA1* was included as an outgroup. Identical amino acids are shaded. (b) Phylogenetic trees were inferred from alignments of RRM sequences and obtained from Bayesian inference. Statistical supports are indicated above (Bayesian posterior probabilities) and below (ML bootstrap values) branches. Values were omitted for clarity in terminal branches. *T. cas*: red flour beetle; *C. pip*, and *A. gam.*: mosquitoes; *L. sal.*: copepod; *O. dio.*: appendicularia; *H. bac.*: parasitic nematode. Protostomes are in light gray background, deuterostomes in dark gray background.

Fox 1, 2, and 3 serum as described in Han et al. (2011) and purified with the gel Melon IgG purification kit (Thermo).

### Animal Husbandry

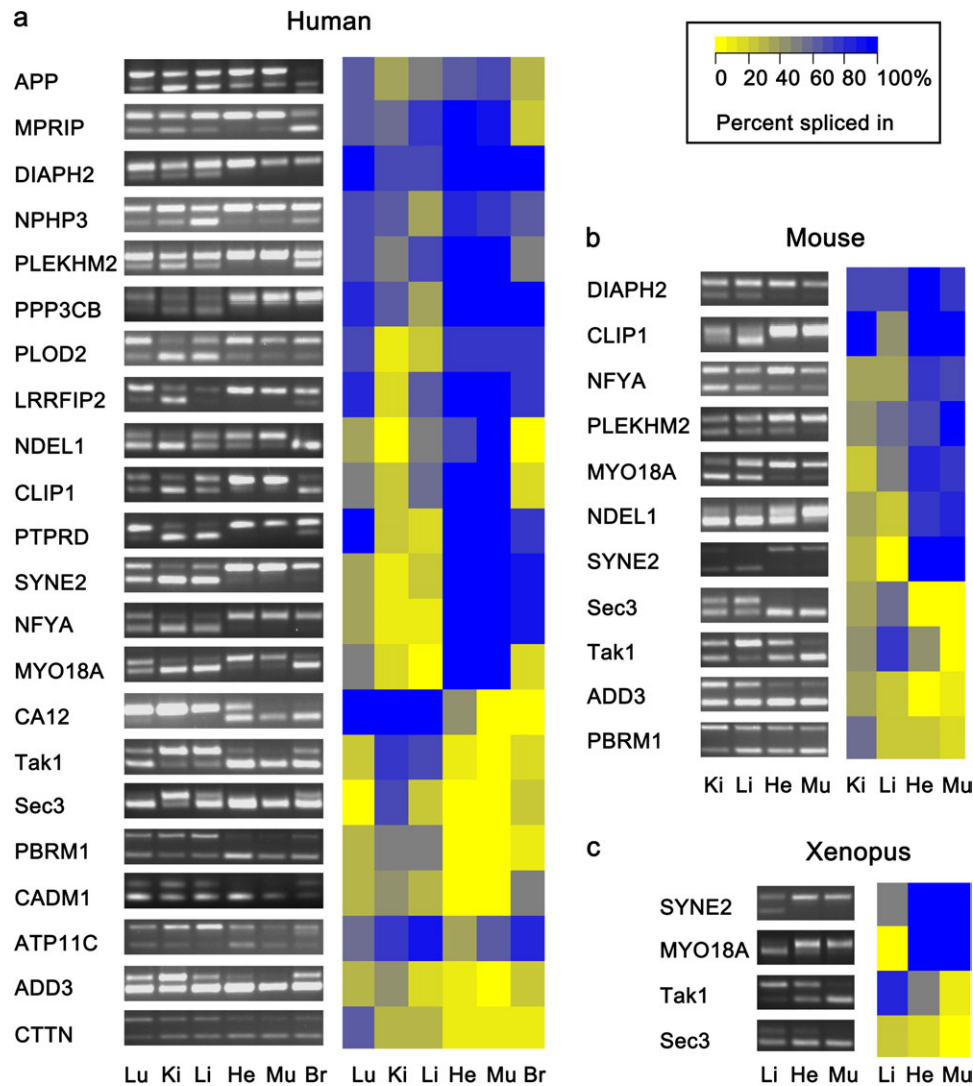
*Xenopus laevis* embryos were obtained by in vitro fertilization, grown as previously described (Vignal et al. 2007) and conventionally staged (Nieuwkoop and Faber 1967). Adults of *C. intestinalis* and *Paracentrotus lividus* were collected in the bay of Roscoff (Finistère) France.

### Results

Splicing factors of the Fox family are highly conserved in vertebrates and ecdysozoans (Kuroyanagi 2009; Damianov and Black 2010). To complete the evolutionary picture, we

sought for Fox orthologs in genomic and mRNA databases. We found Fox-like proteins in all metazoans, from Placozoans (*Trichoplax adhaerens*), but not in the unicellular choanoflagellate *Monosiga brevicollis* (fig. 1). This strong evolutionary conservation in multicellular animals suggests that Fox proteins control basic physiological functions associated with cell differentiation. Interestingly, phylogenetic analysis also revealed a discrepant branching of urochordates, suggestive of a regressive evolution of Fox proteins in this clade.

Fox proteins have the highest binding specificity of known RNA-binding protein splicing factors with a defined binding site UGCAUG. This sequence was found in a genome-wide screen of alternative splicing events to be highly over represented near predicted muscle and heart-specific exons



**Fig. 2.** Tissue-specific alternative splicing of Fox targets in vertebrates. Agarose gels of RT-PCR products using primers either side of the alternatively spliced regions. Upper bands are the long (exon-included) form and lower bands are the short (exon-excluded form). Quantifications of splicing are shown in heat map form on the right of each gel. (a) Twenty-two genes showed tissue-specific splicing regulation ( $>20\%$  on average) between liver and kidney on the one hand and heart and muscle on the other. (b) Eleven of these genes show tissue-specific splicing in mouse. (c) Four genes show tissue-specific splicing in Xenopus (all quantifications, primers used and full gene names are given in [supplementary table S1, Supplementary Material](#) online). Lu, lung; Ki, kidney; Li, liver; He, heart; Mu, skeletal muscle; Br, brain.

correlating with the highest expression of Fox proteins in these tissues (Das et al. 2007; Castle et al. 2008). Many Fox target exons are also highly conserved and the Fox-binding sites near the exons are too ([supplementary fig. S1, Supplementary Material](#) online). However, the conservation of tissue specificity of their splicing patterns has not been investigated. First, we sought to confirm muscle- and heart-specific splicing of Fox substrates; we performed an RT-PCR analysis of the 40 strongest experimentally confirmed Fox targets from a recent study (Venables et al. 2009) ([supplementary figs. S1 and S2, Supplementary Material](#) online). By analyzing six different human tissues, we observed 22 targets showing clear differences ( $>20\%$  on average) in alternative exon inclusion between kidney and liver on the one hand and heart and muscle on the other ([fig. 2a](#) and [supplementary table S1, Supplementary Material](#) online).

We next examined which targets display alternative splicing events conserved across evolution. We analyzed four mouse tissues (kidney, liver, heart, and muscle) and found that 11 of the 22 conserved alternative splicing events showed the same direction of shifts in splicing as had been seen in human ([fig. 2b](#)). We further probed the conservation in frogs. Six target exons were conserved in the frog genome and four displayed tissue-specific alternative splicing in *X. laevis* liver versus heart and muscle: TGF beta associated kinase Tak1 (MAP3K7), Exocyst component Sec3 (ExoC1), Myosin (MYO18A), and SYNE2 ([fig. 2c](#)).

To further probe alternate splicing conservation, we searched available metazoan genomes for potential alternative exons in the four genes. Only the TAK1 gene was identified in all metazoan genomes analyzed ([supplementary table S2, Supplementary Material](#) online); we found exons potentially encoding the Tak1 alternative peptide

**Table 1.** Amino Acid Sequences of Alternative Exons Identified in Tak1 Genes of Selected Deuterostome Species.

Taxons	Species	Alternative Peptide <sup>a</sup>
Placentals	Human	YSKP <u>K</u> RGHRKTASF <u>G</u> NILD----V <u>P</u> EIVIS
	Macaque	YSKP <u>K</u> RGHRKTASF <u>G</u> NILD----V <u>P</u> EIVIS
	Mouse	YSKP <u>K</u> RGHRKTASF <u>G</u> NILD----V <u>P</u> EIVIS
	Rat	YSKP <u>K</u> RGHRKTASF <u>G</u> NILD----V <u>P</u> EIVIS
	Horse	YSKP <u>K</u> RGHRKTASF <u>G</u> NILEMAAYV <u>P</u> EIVIS
Marsupials	Opossum	CSK <u>P</u> KRGHRKTASF <u>G</u> NILD----V <u>P</u> QIVIS
Prototherians	Platypus	YSKP <u>K</u> RGHRKTASF <u>G</u> NILD----V <u>P</u> EIVIS
Sauropsids	Chicken	YSKP <u>K</u> RGHRKTASF <u>G</u> NILD----V <u>P</u> EIIIP
Batrachians	Xenopus	FAK <u>P</u> KRGHRKTASF <u>G</u> NILD----V <u>P</u> EIVIT
Fishes	Zebrafish	RPQ <u>Y</u> KRGHRKTASH <u>G</u> NILD----I <u>P</u> KIIVT
Urochordates	Sea squirt	G <u>P</u> FRKAHRRAS <u>S</u> Y <u>G</u> N <u>T</u> LE----L <u>P</u> YDALR
Cephalochordates	Lancelet	AR <u>P</u> AWQ <u>T</u> HRVR <u>S</u> H <u>G</u> NILD----M <u>I</u> VP <u>Q</u> GS
Echinoderms	Sea urchin (Sp)	KS- <u>A</u> VKS <u>H</u> RRCS <u>H</u> GR <u>I</u> LD----D <u>F</u> P <u>P</u> L <u>P</u> T
	Sea urchin (Sp)	KS- <u>A</u> V <u>Q</u> S <u>H</u> RR <u>S</u> NS <u>H</u> GR <u>I</u> LDQD <u>I</u> P <u>K</u> Y <u>P</u> LP <u>L</u> P <u>I</u>
Hemichordates	Acorn worm 1	<u>P</u> S-- <u>V</u> Q <u>T</u> HR <u>R</u> CR <u>S</u> H <u>G</u> Y <u>I</u> ----- <u>P</u> P <u>Q</u> T <u>F</u> T <u>Y</u>
	Acorn worm 2	T <u>G</u> T <u>F</u> V <u>P</u> G <u>H</u> RR <u>S</u> NS <u>H</u> G <u>N</u> I----- <u>P</u> P <u>Q</u> T <u>Y</u> T <u>S</u>

<sup>a</sup> Amino acids conserved across species are in italics. Proline residues are underlined.

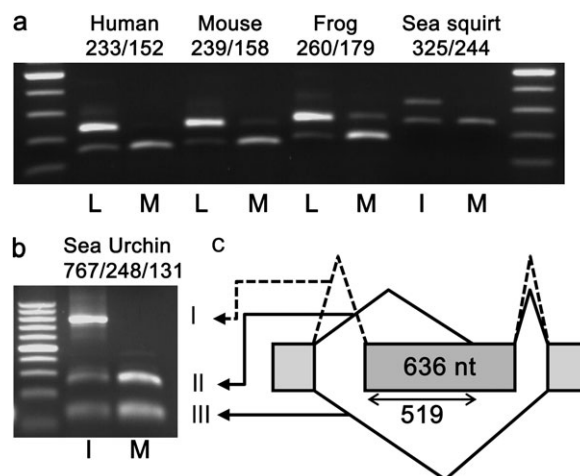
at the expected position in all deuterostome species examined but not for the other three vertebrate-conserved splicing events and no conservation of the alternative peptides could be identified in protostomes (table 1). We then confirmed in the intestine and muscle of the prochordate sea squirt (*C. intestinalis*) that the Tak1 exon identified in silico indeed showed muscle-specific exclusion as had been found in mammals (fig. 3a). Finally, we examined Tak1 expression in sea urchin, where the potential alternatively spliced peptide is included in a 624 nucleotide exon. Like in other deuterostomes, we found the exon was strictly removed in muscle but remained included in intestine (fig. 3b and c). Inhibition of the exon also resulted in some use of a cryptic splice in the exon (supplementary fig. S3, Supplementary Material online).

TAK1 is a crucial effector of the TGF beta-mediated mesoderm induction in *Xenopus* (Ohkawara et al. 2004). Therefore, we looked to see if the Fox-regulated splicing events were altered during *X. laevis* embryonic development. We observed striking temporally regulated exclusion of the alternative exons of Tak1 and Sec3 between the neurula and tadpole stages, concomitant with muscle development (fig. 4a).

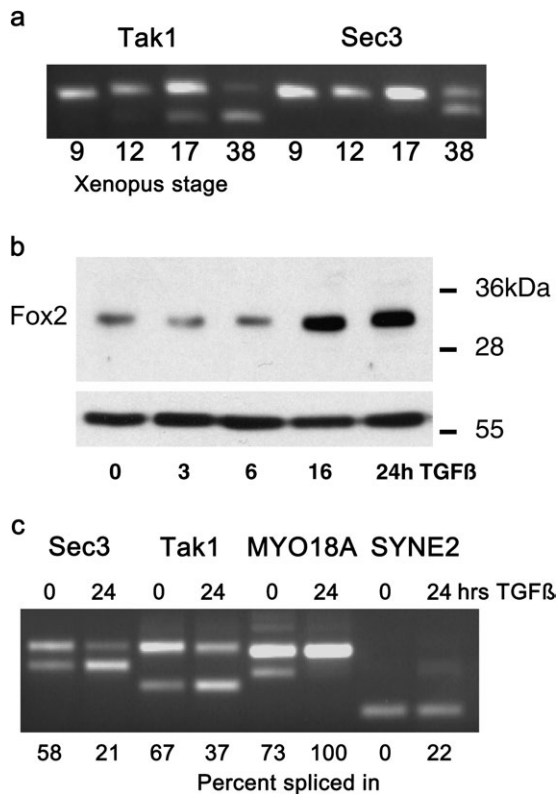
In vitro, TGF beta is the classic cytokine for induction of epithelial to mesenchymal transition (EMT). We therefore used a well-established in vitro model of TGF beta-mediated EMT in mouse NMuMG cells (Bhowmick et al. 2001) to investigate conserved Fox-mediated regulation of alternative splicing. As expected, treatment with 5 ng.ml<sup>-1</sup> TGF beta caused changes indicative of EMT over 24 h (supplementary fig. S4, Supplementary Material online). Western blotting also showed TGF beta caused a strong induction of Fox2 expression (fig. 4b). Consistently, Tak1 splicing was altered after 24-h treatment with TGF beta, with a shift from the long isoform to the short one. This is also in complete agreement with the action of Fox2 as repressor of the inclusion of the Tak1 exon. Consistent with Fox2 upregulation during EMT in NMuMG cells, the four most conserved splicing events (from fig. 2c) were significantly shifted by TGF beta treatment (fig. 4c).

## Discussion

In this study, we demonstrate that a conserved 27 amino acid peptide of TAK1 is excluded through alternative splicing in the muscles of mammals, frog, sea squirt, and sea urchin. Although conservation of alternatively spliced exons has been documented in vertebrates (Jelen et al. 2007), Tak1 represents the first example of conserved tissue-regulated alternative splicing in deuterostomes. Other candidate genes were the fibroblast growth factor FGFR2, shown to use two



**Fig. 3.** Muscle-specific splicing of Tak1 is conserved in deuterostomes. (a) A short exon of 81 nucleotides is specifically removed from muscle Tak1 transcripts in four chordate species (Frog: *Xenopus laevis*, Sea squirt: *Ciona intestinalis*). (b) A longer exon coding for a similar peptide is specifically removed in sea urchin (*Paracentrotus lividus*) muscle Tak1 transcripts. (c) Cartoon outlining the three alternative transcripts identified in sea urchin Tak1. The novel PCR products in b were sequenced (GenBank accession numbers JF342573–4). The shortest product corresponds to the absence of the 636 nucleotides exon, and the intermediate product results from the use of a cryptic 3' splice site located 519 nucleotides downstream in the large exon. The exact position of the novel splice junction is indicated in (supplementary figure S3 Supplementary Material online). PCR product sizes are indicated. (L) liver, (M) muscle, (I) intestine.



**Fig. 4.** Alternative splicing of Fox targets Tak1 and Sec3 is regulated during development and by TGF beta. (a) Agarose gel showing the splicing of Tak1 and Sec3 at successive stages in the frog *Xenopus laevis* during embryonic development. Fertilized eggs were allowed to develop until stages 9 (blastula), 12 (gastrula), 17 (neurula), and 38 (tadpole). (b) Western blot demonstrating induction of Fox2 around 16–24 h after treatment of mouse NMuMG cells with 5 ng/ml TGF beta (the cells, which underwent an EMT, are shown in [supplementary fig. S4, Supplementary Material](#) online). (c) Agarose gel showing the result of 24 h of TGF beta treatment on the splicing of the four most conserved splicing events in mouse NMuMG cells.

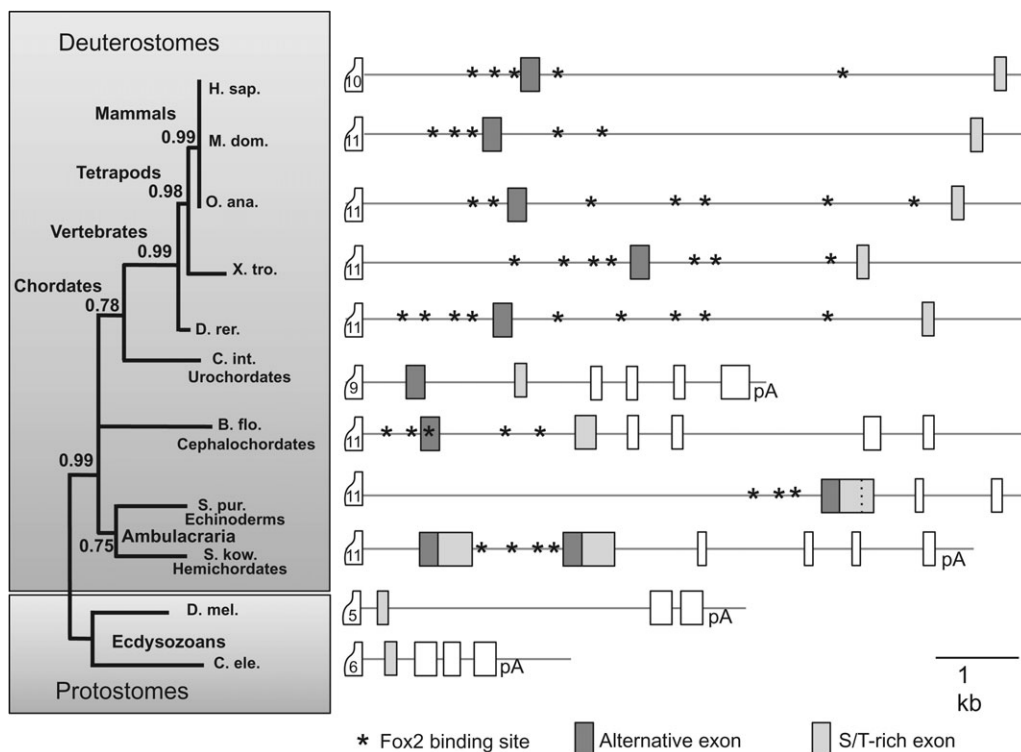
mutually exclusive exons in *C. elegans* and mammals depending on cellular states and Tra2, whose alternative splicing was reported in *Drosophila* and man (McGuffin et al. 1998; Kuroyanagi 2009). In Tra2, an intron is regulated in flies, whereas an exon is regulated in mammals. In FGFR2, the mutually exclusive exons are in different parts of the protein in deuterostomes and protostomes, so these are likely examples of alternative splicing evolution converging on common genes and do not represent orthologous splicing events. However, the alternative splicing of exons IIIb and IIIc of FGFR2 occurred relatively early on in deuterostome evolution (Bhatnagar et al. 2010), although the sea urchin has only one exon in this region and we found no evidence of alternative splicing of this exon in four sea urchin tissues (data not shown).

In chordates, the regulated exon in Tak1 is of a fixed size of 81 nucleotides (except the horse exon, which codes an extra four amino acids, see [table 1](#) and [supplementary fig. S1, Supplementary Material](#) online), whereas in the Ambulacraria clade, which includes hemichordates and echinoderms (Turbeville et al. 1994), the peptide is encoded by

the 5' end of a much larger exon ([fig. 5](#)). In hemichordates (acorn worm), two Tak1 isoforms might be generated from a tandem-repeated cassette containing the alternative 27 amino acids, which supports a scenario in which the Tak1 gene ancestor in deuterostomes already contained the alternatively spliced exon. Furthermore, we observed a preponderance of Fox-binding sites in the intron upstream of the alternative exon in all species except *Ciona* suggesting that Fox proteins already controlled Tak1 gene alternative splicing in early deuterostome evolution ([fig. 5](#)). The absence of the hexameric Fox-binding sites upstream of the alternatively spliced Tak1 exon and the divergence of the RRM domain of Fox ([fig. 1](#)) are features shared by both *C. intestinalis* and *C. savignyi* species (Data not shown), which suggests a relaxation of the constraints exerted on the two genes. However, we found low  $K_a/K_s$  ratios between Fox and Tak1 coding sequences of *C. intestinalis* and *C. savignyi* (below 0.07 whatever the substitution model,  $P$  value  $< 5.4 \times 10^{-57}$ , [supplementary table S4, Supplementary Material](#) online). This indicates that the two genes are still under strong purifying selection in *Cionidae* and suggests that the observed relaxation likely affects the functional link between the Fox and Tak1 genes. In this respect, *Cionidae*, thus appear more similar to insects and nematodes ([fig. 1b](#)), in which there is no Tak1 alternative exon that could be controlled by Fox proteins ([fig. 5](#)).

Previous studies have suggested, that for the most part, alternative splicing regulators are highly conserved in evolution, but that these conserved regulators acquire new target exons in a species-specific manner (Jelen et al. 2007; Brooks et al. 2011; Irimia et al. 2011). Indeed, only one-third of human alternative splicing events can be found in mouse (Mudge et al. 2011). Our finding of conserved regulation of only half of the 22 Fox target exons between human and mouse is consistent with this modest conservation ([fig. 2b](#)); however, the extreme conservation of Tak1 alternative splicing in sea squirt ([fig. 2a](#)) and the disappearance of the Fox elements in this species ([fig. 5](#)) imply that some key alternative splicing events have essential developmental functions, whereas the upstream genes controlling them may vary, as has been observed for the alternative splicing cascade controlling sex determination in flies (Shukla and Nagaraju 2010). An alternative possibility is that the urochordate Fox proteins have acquired an altered RNA-binding specificity that is mirrored in their Tak1 transcript.

Core components of the TGF beta pathway were shown recently to have emerged in early metazoans (Huminiacki et al. 2009). Our study extends this early emergence to Tak1 and to RNA-binding proteins of the RBFOX family. Furthermore, the fact that Fox2 is induced upon TGF beta treatment together with the timing of occurrence of the Tak1 alternative exon strongly suggests that deuterostomes have selected Fox2-dependent alternative splicing as an integral part of TGF beta signaling. This may also be the case for other physiological functions of TGF beta, such as chondrogenic differentiation in which the SRp40 splicing factor is induced (Han et al. 2007) or angiogenesis, in which TGF beta induces a switch in VEGF isoforms that depends on the splicing factor SRp55 (Nowak et al. 2008).



**Fig. 5.** Tak1 alternative exons and Fox-binding sites are conserved in deuterostomes. On the left is a Bayesian phylogenetic tree inferred from amino acid alignment of TAK1 kinase domains showing posterior probabilities for each node. On the right, the exon–intron structure of each Tak1 gene is shown starting from the last exon of the kinase domain (numbered). Exonic sequences encoding the alternative peptide are in dark gray; those encoding serine/threonine-rich regions are in light gray. The dotted line in *Strongylocentrotus purpuratus* exon 12 corresponds to the position of the cryptic acceptor site identified in *Paracentrotus lividus* mRNAs (see fig. 3b and supplementary fig. S3, Supplementary Material online). Fox2-binding sequences (UGCAUG) are shown by asterisks.

Sequence analysis of TAK1 alternative peptides showed highly conserved features from acorn worm to man; peptides are flanked by prolines and contain basic residues, potential sites for PKA- or CK1-dependent phosphorylation, as well as putative binding sites, and PDZ, SH2, and PP2B domains (<http://elm.eu.org/>, data not shown). Several TAK1-binding proteins (TABs) have already been reported, whose implication in TAK1 signaling have been largely documented: TAB1, interacting with the kinase domain at the N-terminus (Yamaguchi et al. 1999), TAB2 and TAB3 that interact with the C-terminal coiled-coil domain (Takaesu et al. 2000) and TAB4/TIP41, a negative regulator of the TOR pathway (Jalcinto et al. 2001), which also binds to the central region of TAK1 (Prickett et al. 2008). TAB proteins are highly conserved across evolution, especially TAB4 which is found in all eukaryotes (supplementary table S5, Supplementary Material online). Interestingly, the TAK1 alternative peptide is located near the TAB4-binding region directly adjacent to a residue that is phosphorylated upon TAB4 binding. Loss of the alternative peptide in TGF beta-treated cells might thus modify the distribution of TAK1 complexes and induce different cellular outcomes.

In conclusion, this study emphasizes the use of evolutionary studies to identify conserved regulatory features. The conservation of the Tak1 alternative splicing pattern in deuterostomes therefore suggests that this feature adapts

tissue-specific cell physiology to external signaling and implies a major functional difference between the TAK1 isoforms which calls for further investigation.

## Supplementary Material

Supplementary figures S1–S4 and tables S1–S5 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

## Acknowledgments

J.P.V. and J.T. were supported by the European Alternative Splicing Network of Excellence (EURASNET, FP6 life sciences, genomics and biotechnology for health) and a grant from Canceropôle and l'INCa 2009-1-RT-10-CNRS13-1. J.T. was supported by Institut Universitaire de France as a senior member. This work was also supported by centre national pour la recherche scientifique institutional grants. E.V. and P.F. were supported by ligue nationale contre le cancer's Aude and Herault committee and Association pour la recherche contre le cancer grant number 1048. Thanks to Julien Gervais-Bird for help with the study design and to Sachiyo Kawamoto (National Heart, Lung and Blood Institute/National Institute of Health) for the kind contribution of anti mouse-Fox 1, 2, and 3 sera.



## References

- Abascal F, Zardoya R, Posada D. 2005. ProtTest: selection of best-fit models of protein evolution. *Bioinformatics* 21:2104–2105.
- Bhatnagar S, Kumar A, Makonchuk DY, Li H. 2010. Transforming growth factor-beta-activated kinase 1 is an essential regulator of myogenic differentiation. *J Biol Chem*. 285:6401–6411.
- Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, Arteaga CL, Moses HL. 2001. Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol Biol Cell*. 12: 27–36.
- Black DL. 2003. Mechanisms of alternative pre-messenger RNA splicing. *Annu Rev Biochem*. 72:291–336.
- Bland CS, Wang ET, Vu A, David MP, Castle JC, Johnson JM, Burge CB, Cooper TA. 2010. Global regulation of alternative splicing during myogenic differentiation. *Nucleic Acids Res*. 38:7651–7664.
- Brooks AN, Yang L, Duff MO, Hansen KD, Park JW, Dudoit S, Brenner SE, Graveley BR. 2011. Conservation of an RNA regulatory map between *Drosophila* and mammals. *Genome Res*. 21:193–202.
- Castle JC, Zhang C, Shah JK, Kulkarni AV, Kalsotra A, Cooper TA, Johnson JM. 2008. Expression of 24,426 human alternative splicing events and predicted cis regulation in 48 tissues and cell lines. *Nat Genet*. 40:1416–1425.
- Damianov A, Black DL. 2010. Autoregulation of Fox protein expression to produce dominant negative splicing factors. *RNA*. 16:405–416.
- Das D, Clark TA, Schweitzer A, et al. (11 co-authors). 2007. A correlation with exon expression approach to identify cis-regulatory elements for tissue-specific alternative splicing. *Nucleic Acids Res*. 35:4845–4857.
- Dempsey CE, Sakurai H, Sugita T, Guesdon F. 2000. Alternative splicing and gene structure of the transforming growth factor beta-activated kinase 1. *Biochim Biophys Acta*. 1517:46–52.
- Elliott DJ, Ladomery M. 2011. Molecular biology of RNA. New York: Oxford University Press.
- Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol*. 59:307–321.
- Han F, Gilbert JR, Harrison G, et al. (12 co-authors). 2007. Transforming growth factor-beta1 regulates fibronectin isoform expression and splicing factor SRp40 expression during ATDC5 chondrogenic maturation. *Exp Cell Res*. 313:1518–1532.
- Han J, Ding JH, Byeon CW, Kim JH, Hertel KJ, Jeong S, Fu XD. 2011. SR proteins induce alternative exon skipping through their activities on the flanking constitutive exons. *Mol Cell Biol*. 31:793–802.
- Huelsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17:754–755.
- Huminięcki L, Goldovsky L, Freilich S, Moustakas A, Ouzounis C, Heldin CH. 2009. Emergence, development and diversification of the TGF-beta signalling pathway within the animal kingdom. *BMC Evol Biol*. 9:28.
- Irimia M, Denuc A, Burguera D, et al. (11 co-authors). 2011. Stepwise assembly of the Nova-regulated alternative splicing network in the vertebrate brain. *Proc Natl Acad Sci U S A*. 108:5319–5324.
- Jacinto E, Guo B, Arndt KT, Schmelzle T, Hall MN. 2001. TIP41 interacts with TAP42 and negatively regulates the TOR signaling pathway. *Mol Cell*. 8:1017–1026.
- Jelen N, Ule J, Zivin M, Darnell RB. 2007. Evolution of Nova-dependent splicing regulation in the brain. *PLoS Genet*. 3:1838–1847.
- Kalsotra A, Xiao X, Ward AJ, Castle JC, Johnson JM, Burge CB, Cooper TA. 2008. A postnatal switch of CELF and MBNL proteins reprograms alternative splicing in the developing heart. *Proc Natl Acad Sci U S A*. 105:20333–20338.
- Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res*. 30:3059–3066.
- Kondo M, Osada H, Uchida K, Yanagisawa K, Masuda A, Takagi K, Takahashi T. 1998. Molecular cloning of human TAK1 and its mutational analysis in human lung cancer. *Int J Cancer*. 75:559–563.
- Kuroyanagi H. 2009. Fox-1 family of RNA-binding proteins. *Cell Mol Life Sci*. 66:3895–3907.
- Li MG, Katsura K, Nomiyama H, Komaki K, Ninomiya-Tsuji J, Matsumoto K, Kobayashi T, Tamura S. 2003. Regulation of the interleukin-1-induced signaling pathways by a novel member of the protein phosphatase 2C family (PP2Cepsilon). *J Biol Chem*. 278:12013–12021.
- Massague J. 2008. TGFbeta in cancer. *Cell* 134:215–230.
- McGuffin ME, Chandler D, Somaiya D, Dauwalder B, Mattox W. 1998. Autoregulation of transformer-2 alternative splicing is necessary for normal male fertility in *Drosophila*. *Genetics* 149:1477–1486.
- Mudge JM, Frankish A, Fernandez-Banet J, Alioto T, Derrien T, Howald C, Reymond A, Guigo R, Hubbard T, Harrow J. Forthcoming 2011. The origins, evolution and functional potential of alternative splicing in vertebrates. *Mol Biol Evol*.
- Nieuwkoop PD, Faber J. 1967. Normal table of *Xenopus laevis*. Amsterdam (The Netherlands): North-Holland Publishing Company.
- Nowak DG, Woolard J, Amin EM, Konopatskaya O, Saleem MA, Churchill AJ, Ladomery MR, Harper SJ, Bates DO. 2008. Expression of pro- and anti-angiogenic isoforms of VEGF is differentially regulated by splicing and growth factors. *J Cell Sci*. 121:3487–3495.
- Ohkawara B, Shirakabe K, Hyodo-Miura J, Matsuo R, Ueno N, Matsumoto K, Shibuya H. 2004. Role of the TAK1-NLK-STAT3 pathway in TGF-beta-mediated mesoderm induction. *Genes Dev*. 18:381–386.
- Prickett TD, Ninomiya-Tsuji J, Broglie P, Muratore-Schroeder TL, Shabanowitz J, Hunt DF, Brautigan DL. 2008. TAB4 stimulates TAK1-TAB1 phosphorylation and binds polyubiquitin to direct signaling to NF-kappaB. *J Biol Chem*. 283:19245–19254.
- Shibuya H, Iwata H, Masuyama N, Gotoh Y, Yamaguchi K, Irie K, Matsumoto K, Nishida E, Ueno N. 1998. Role of TAK1 and TAB1 in BMP signaling in early *Xenopus* development. *EMBO J*. 17:1019–1028.
- Shukla JN, Nagaraju J. 2010. Doublesex: a conserved downstream gene controlled by diverse upstream regulators. *J Genet*. 89:341–356.
- Takaesu G, Kishida S, Hiyaama A, Yamaguchi K, Shibuya H, Irie K, Ninomiya-Tsuji J, Matsumoto K. 2000. TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. *Mol Cell*. 5:649–658.
- Turbeville JM, Schulz JR, Raff RA. 1994. Deuterostome phylogeny and the sister group of the chordates: evidence from molecules and morphology. *Mol Biol Evol*. 11:648–655.
- Venables JP, Klinck R, Koh C, et al. (17 co-authors). 2009. Cancer-associated regulation of alternative splicing. *Nat Struct Mol Biol*. 16:670–676.
- Vignal E, de Santa Barbara P, Guemar L, Donnay JM, Fort P, Faure S. 2007. Expression of RhoB in the developing *Xenopus laevis* embryo. *Gene Expr Patterns*. 7:282–288.
- Yamaguchi K, Nagai S, Ninomiya-Tsuji J, Nishita M, Tamai K, Irie K, Ueno N, Nishida E, Shibuya H, Matsumoto K. 1999. XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway. *EMBO J*. 18:179–187.
- Yeo GW, Coufal NG, Liang TY, Peng GE, Fu XD, Gage FH. 2009. An RNA code for the FOX2 splicing regulator revealed by mapping RNA-protein interactions in stem cells. *Nat Struct Mol Biol*. 16:130–137.

Zhang C, Zhang Z, Castle J, Sun S, Johnson J, Krainer AR, Zhang MQ. 2008. Defining the regulatory network of the tissue-specific splicing factors Fox-1 and Fox-2. *Genes Dev.* 22:2550–2563.

Zhang Z, Li J, Zhao XQ, Wang J, Wong GK, Yu J. 2006. KaKs\_Calculator: calculating Ka and Ks through model selection and model averaging. *Genomics Proteomics Bioinformatics.* 4:259–263.