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## Expression and evolution of Tiki1 and Tiki2 genes in vertebrates

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### Abstract

Tiki1 is a Wnt protease and antagonist specifically expressed in the Spemann-Mangold Organizer and is required for head formation in *Xenopus* embryos. Here we report neighbor-joining phylogenetic analysis of vertebrate Tiki genes and their mRNA expression patterns in chick, mouse, and rabbit embryos. Tiki1 and Tiki2 orthologues are highly conserved, and exhibit similar but also different developmental expression patterns among the vertebrate/mammalian species analyzed. The *Tiki1* gene is noticeably absent in the rodent lineage, but is present in lagomorphs and all other vertebrate/mammalian species examined. Expression in Hensen's node, the equivalent of the *Xenopus* Organizer, was observed for Chick *Tiki2* and Rabbit *Tiki1* and *Tiki2*. Mouse *Tiki2* was detected at low levels at gastrulation and head fold stages, but not in the node. Mouse *Tiki2* and chick *Tiki1* display similar expression in the dorsal spinal cord. Chick *Tiki1* expression was also detected in the surface ectoderm and maxillary bud, while chick *Tiki2* was found in the anterior intestinal portal, head mesenchyme and primitive atrium. Our expression analyses provide evidence that *Tiki1* and *Tiki2* are evolutionary conserved among vertebrate species and their expression in the Organizer and other regions suggests contributions of these Wnt inhibitors to embryonic patterning as well as organogenesis. Our analyses further reveal misregulation of TIKI1 and TIKI2 in human cancer and diseases.

### Keywords

Tiki1; Tiki2; Wnt; organizer; head induction; organogenesis

### INTRODUCTION

The Wnt signaling pathway plays central roles in a multitude of biological processes, including embryogenesis, tissue homeostasis, and regeneration, and its perturbation causes degenerative diseases, birth defects and cancer (Clevers and Nusse, 2012, MacDonald *et al.*,

2009). In addition to the existence of a large family of the secreted Wnt ligands, Wnt signaling is also controlled by several families of secreted and membrane-tethered Wnt modulators, which individually or in concert regulate the Wnt pathway activity spatially and temporarily as best exemplified during antero-posterior (AP) patterning in early vertebrate embryogenesis (Cruciat and Niehrs, 2013). During gastrulation of *Xenopus* embryos, high Wnt activity promotes posterior patterning whereas low or no Wnt activity is required for anterior head formation (MacDonald *et al.*, 2009, Niehrs, 2004, Petersen and Reddien, 2009). Precise regulation is achieved by several classes of Wnt antagonists that are specifically expressed in the Spemann-Mangold organizer and its descendent tissues in the anterior anlagen, including secreted Frizzled-related proteins (sFRPs), cerberus, Dickkopf-1 (Dkk1), and Tiki1 (De Robertis and Kuroda, 2004, Niehrs, 2004, Zhang *et al.*, 2012). Anterior inhibition of the Wnt pathway is essential for establishing AP patterning throughout vertebrate species, although the complement of Wnt antagonists expressed in the Organizer may differ among species (Niehrs, 2004).

Tiki1 is the newest Wnt antagonist identified that is specifically expressed in the Organizer and the descendent prechordal mesendoderm, and is required for head development in *Xenopus* embryos (Zhang *et al.*, 2012). Tiki1, and its paralogue Tiki2, are found in most vertebrates and encode metalloproteases that cleave Wnt proteins at the amino terminus thereby inactivating Wnt morphogens (Zhang *et al.*, 2012). Wnt inactivation by Tiki reveals an exquisite regulatory system that acts in both Wnt-secreting and Wnt-responding cells (Zhang *et al.*, 2012). Considering *Tiki1* expression and function during *Xenopus* embryogenesis and the broader role of Wnt signaling in AP patterning in vertebrates, we identified and performed evolutionary sequence analysis of Tiki1 and Tiki2 in vertebrate/mammalian species, and carried out comparative *in situ* hybridization of Tiki1 and Tiki2 in avian, rabbit and mouse embryos. Our results show an early anterior Tiki gene expression in the vertebrate/mammalian organizer suggesting a conserved role in AP patterning, with the exception of the rodent lineage, which has lost the Tiki1 gene.

## RESULTS

### Evolutionary conservation of Tiki1 and Tiki2 among vertebrates

Phylogenetic analysis using TIKI protein sequences was performed using a Neighbor-Joining method, which is suitable for reconstructing phylogenetic trees using evolutionary distance data. This method has advantages in that it is fast and thus suited for large datasets and bootstrap analysis, and permits delineation of lineages with different branch lengths and correction for multiple substitutions (Saitou and Nei, 1987). Our phylogenetic analyses revealed that Tiki proteins are highly conserved among vertebrate species (Fig. S1). All species analyzed have both *Tiki1* and *Tiki2* genes, except for Rodentia, which appear to have lost *Tiki1* (Fig. 1A). The *Tiki1* gene spans seven exons in the human and rabbit genomes (Fig. S2). Remnants of the ancient *Tiki1* gene could be detected in the rodent genomes (mouse, rat, guinea pig and squirrel) in a region that is syntenic with the *Tiki1* locus in human and rabbits; however the residual exon fragments no longer encode a full-length Tiki1 protein (Fig. S2). Curiously, the group most closely related to Rodentia, the Lagomorpha (rabbit), has *Tiki1* and *Tiki2*, suggesting that this loss has occurred exclusively

in Rodentia. In addition to the common rabbit (*Oryctolagus cuniculus*), supporting evidence for the presence of *Tiki1* and *Tiki2* in Lagomorpha is also found in the draft genome sequence of the Pika rock rabbit (*Ochotona princeps*) (data not shown).

Human TIKI1 displays lower sequence identity (numbers in red) with its mammalian orthologs (red) compared to that of human TIKI2 with its orthologs (Fig. 1B). Surprisingly Chicken and *Xenopus* Tiki1 proteins are more similar (green) and thus grouped together compared to the orthologous Chicken and *Xenopus* Tiki2 (Fig. 1B), possibly reflecting an evolutionary gap as a result of the loss of the *Tiki1* gene in rodents. Given the loss of *Tiki1* in Rodentia, which shared the last common ancestor with humans ~90MYA, we analyzed the orthologous conservation of Tiki1 and Tiki2 within mammals. A pair-wise comparison between *TIKI* genes from Human, Macaque, and Rabbit show that *TIKI2* exhibits higher orthologous (interspecies) conservation than *TIKI1* (Fig. S1). The greater divergence in mammalian Tiki1 proteins and the loss of Tiki1 in the rodent lineage suggests that *Tiki2* may be under stronger selective pressure than *Tiki1* during mammalian evolution.

### Expression of Tiki genes during chick development

The chick embryo develops as a bilayer blastodisc with an epiblast and underlying hypoblast in a manner similar to embryogenesis of humans and most mammals (Solnica-Krezel and Sepich, 2012, Viebahn, 1999). The expression pattern of *Tiki1* and *Tiki2* was examined in chick embryos from HH4 to E3 (HH20-21). The earliest stage we detected *Tiki1* expression is at HH4 (Fig. 2A). *Tiki1* is expressed within embryonic tissues in the area pellucida/area opaca boundary, in the anterior, lateral and posterior regions of the area pellucida (Fig. 2A, arrows). Cross section images show that *Tiki1* mRNA is present in both epiblast and hypoblast (Fig. 2A'; asterisks). At HH5 a diffuse and ubiquitous expression of *Tiki1* was faintly detected (Fig. 2B). Later on at the 3 somite stage (ss; HH8) we did not detect any significant *Tiki1* expression (Fig. 2C and D). At the 6ss *Tiki1* becomes detectable in the cephalic region and lateral to Hensen's node (Fig. 2E). Its expression was specifically found in the surface ectoderm and at the lateral plate mesoderm of the pericardial portion of the pleural-peritoneal cavity (Fig. 2E, E'). The neural tube, notochord, mesenchyme and pharyngeal endoderm were negative for *Tiki1* expression (Fig. 2E'). At E2 (embryonic day 2) no expression of *Tiki1* was detected (Fig. 2F and G). However, from E2.5 *Tiki1* transcripts were found in the lateral forehead ectoderm (Fig. 2H arrowhead) and in the dorsal neural tube from the myelencephalon to more posterior regions (Fig. 2H' and H''). At E3 *Tiki1* expression could be detected in the ectoderm of the maxillary bud (Fig. 2I, I'; black arrow). The expression is maintained in the neural tube along the trunk axis (Fig. 2I, J and J'; white arrows), but not in the cervical and more anterior regions (Fig. 2I and J, black arrowheads). Moreover, *Tiki1* is not expressed in the caudal neural tube posterior to the hind limb bud (Fig. 2J''). *Tiki1* expression is restricted to the dorsal part of neural tube as observed in cross sections of the trunk region of an E3 chick embryo (Fig. 2J''').

*Tiki2* mRNA was observed in the germinal crescent (Fig. 3A, arrows) and in Hensen's node of the HH4 chick embryo (Fig. 3A). Interestingly, at HH5 *Tiki2* is expressed in the prechordal plate (PcP) and the endoderm but not the ectoderm (Fig. 3B, B'). In the endoderm *Tiki2* expression is medial though not extended laterally (Figure 3B', arrowhead).

*Tiki2* is expressed in Hensen's node, PcP and medial endoderm (Fig. 3B''). At the 4ss *Tiki2* mRNA was detected throughout the cephalic region, and in the anterior intestinal portal (AIP) of the foregut (Fig. 3C and C'). At the 5ss *Tiki2* is expressed laterally and ventrally to the anterior neural tube (Fig. 3D, D'). At 7ss we detected *Tiki2* in the head mesenchyme but not in the ectoderm and neural tube, although we observed a slight expression in the neural ectodermal ridge (Fig. 3E black arrow). *Tiki2* expression persists at the 9ss and was detected ubiquitously in the cephalic region up to AIP (Fig. 3F). In order to describe *Tiki2* expression more precisely, cross sections in the cephalic region were examined along the AP axis (Fig. 3F, dashed lines). In the mesencephalic region *Tiki2* mRNA was observed in the head mesenchyme (white arrow), in the pharyngeal endoderm (En) and lateral/ventral ectoderm (asterisks; Fig. 3F'). At the level of the anterior rhombencephalon, *Tiki2* expression was detected in the paraxial mesoderm (empty arrows), lateral plate mesoderm (empty arrowheads), pharyngeal endoderm (En), and lateral/ventral ectoderm (asterisks) (Fig. 3F''). In the posterior rhombencephalon at the otic vesicle level, *Tiki2* expression was detected in the lateral plate mesoderm (empty arrowheads) and pharyngeal endoderm (En) (Fig. 3F'''). In more posterior cross sections *Tiki2* mRNA is expressed in the lateral plate mesoderm, the adjacent endoderm and the dorsal endoderm (Fig. 3F''', arrows). We did not detect any *Tiki2* expression in the somites during our analysis (Fig. 3F'''). At E2 *Tiki2* was detected at the AIP and primitive atrium (Fig. 3G, G'). At E3.5 *Tiki2* mRNA is restricted to the ectoderm dorsal and lateral to the neural tube and in lateral mesoderm between somites (Fig. 3H, I and I'; arrowheads).

### **Tiki1 and Tiki2 expression during early rabbit development**

We next examined the expression pattern of *Tiki1* and *Tiki2* by whole-mount *in situ* hybridization in rabbit embryos during gastrulation and neurulation. Rabbit embryos are flat and disc-shaped and resemble human embryonic development more closely than do mouse embryos, which develop via an inverted cup-shaped "egg cylinder" instead of a flat blastodisc (Solnica-Krezel and Sepich, 2012, Viebahn, 1999). At about day 7.5 post coitum, formation of Hensen's node (the equivalent of Spemann's Organizer) at the rostral end of the elongated primitive streak is the landmark of gastrulation stage 4. At this time, implantation events are initiated (Idkowiak *et al.*, 2004).

*Tiki1* was first detected when Hensen's node appears at the tip of the primitive streak at stage 4<sup>-</sup> (Fig. 4A). At this stage, *Tiki1* expression is restricted to cells of the hypoblast layer. This expression is localized to an anterior crescent-shaped domain from which faint staining fans out posteriorly (Fig. 4A'). Additionally, *Tiki1* is expressed in the hypoblast underlying Hensen's node and its periphery (Fig. 4A''). At stage 4<sup>+</sup>, when the primitive streak is fully elongated, the crescent-shaped domain of *Tiki1* vanishes while its expression becomes restricted to an area encircling Hensen's node (Fig. 4B). Concomitant with the elongation of the notochordal process and emergence of the notochordal plate at stage 5, circular expression of *Tiki1* around Hensen's node becomes elongated and expanded along the AP axis (Fig. 4C), and *Tiki1* was detected in the PcP region (Fig. 4C') as well as in endodermal tissue flanking the notochordal plate on either side (Fig. 4C''). At stage 6, *Tiki1* expression has ceased in cells bordering the notochordal plate (Fig. 4D). Instead, the entire endoderm of the posterior half of the embryo now expresses *Tiki1* (Fig. 4D'). During somitogenesis, *Tiki1*

expression appears in the developing heart, such that at the four somite stage, transcripts are found in endodermal cells all along the crescent-shaped heart anlage (Fig. 4E, E'). For the first time, *Tiki1* is also expressed in cells other than hypoblast/endoderm, as it appears in both primitive streak and presomitic mesoderm (Fig. 4E''). Expression of *Tiki2* becomes apparent at stage 4<sup>+</sup>, when transcripts are distributed uniformly in all three germ layers all across the embryonic blastodisc (Fig. 4F, F'). A localized pattern of *Tiki2* emerges during somitogenesis (Fig. 4G). In embryos at the six somite stage, anterior-most expression of *Tiki2* is found in mesendodermal cells of the prechordal plate (Fig. 4G'). Transcripts are absent from the heart (Fig. 4G''), however, cells of the intermediate mesoderm, caudally abutting the heart anlage, expressed *Tiki2* (Fig. 4G'''). A conspicuous pattern of *Tiki2* is present in presomitic and somitic mesoderm, with highest expression of *Tiki2* in the latest formed (black arrowhead in Fig. 4G; Fig. 4G''') and slightly lower in the currently condensing somite (outlined arrowhead in Fig. 4G).

### **Tiki2 expression during mouse development**

As aforementioned all sequenced vertebrate and mammalian (including human) genomes contain two *Tiki* genes, with the only exception of rodents, which do not have *Tiki1* (Fig. 1). One speculation is that *Tiki1* was selectively lost in the rodent lineage during evolution correlating, in part, with its unique early development as an inverted egg cylinder rather than a hypoblastic disc as in humans and other mammals (Arkell and Tam, 2012, Solnica-Krezel and Sepich, 2012, Viebahn, 1999). Following the loss of the rodent *Tiki1* gene (after the Lagomorph/Rodent divergence ~60 MYA) (Benton and Donoghue, 2007) it is possible that novel expression patterns of *Tiki2* may have evolved to compensate for *Tiki1* function. Therefore we examined the expression of *Tiki2* during early and mid gestational development in mice. *Tiki2* expression is absent from pre-streak embryos (Fig. 5A) and during the early phase of gastrulation (Fig. 5B). *Tiki2* was first detected in the extraembryonic tissues following the closure of the amnion (Fig. 5C), followed by a broad anterior expression prominent at the neural folds (Fig. 5D-F). Mouse *Tiki2* was present in the early heart anlagen before fusion of the right and left primitive aortae (Fig. 5F) and outflow tracts (Fig. 5G and G'). Expression in the developing heart persisted as the highest *Tiki2* expression domain from E8.0 until embryo turning and neural tube closure (Fig. 5G-I). Rings of *Tiki2* expression were detected later in development (Fig. 5L-M). *Tiki2* is also highly expressed in the PcP (Fig. 5H'). A dorsal stripe of *Tiki2* expression was observed from the rostral prosencephalon to the rhombencephalon (Fig. 5J-M). Prosencephalon expression of *Tiki2* became more elaborate at the olfactory placode (Fig. 5L-M). *Tiki2* was absent from the limb buds during the stages we examined, however *Tiki2* was expressed along the lateral plate mesoderm between the fore and hind limb buds (Fig. 5N). *Tiki2* displayed an elaborate expression pattern in the intermediate mesoderm and developing spinal cord (Fig. 5N-O).

We also examined mouse *Tiki2* expression in several adult tissues via northern blotting. Transcripts of 6.7kb, 5.0kb and 2.2kb were detected using the full-length *Tiki2* 1.5kb cDNA probe. These sizes are consistent with predicted polyadenylation sites and represented by ESTs from oligo dT-primed libraries. *Tiki2* displayed the highest expression in adult heart

and testis, followed by the brain, lung and kidney. These results are consistent with an earlier report that high *Tiki2* expression was detected in the heart and kidney (Zhang, 2012).

## Discussion

### Tiki proteins in vertebrates/mammals

The Tiki family constitutes a new class of Wnt signaling antagonists, operating via proteolytic cleavage of the amino terminus of, and thereby inactivating, Wnt proteins (Zhang *et al.*, 2012). A Tiki homologue is encoded in the genome of the sea anemone *Nematostella* and of the sponge *Amphimedon*, suggesting that the Wnt-inhibitory action of Tiki proteins likely evolved early in metazoans before the chordate gene duplication event that generated Tiki1 and Tiki2 (Zhang *et al.*, 2012). Our phylogenetic analysis shows that *Tiki1* and *Tiki2* orthologues are highly conserved among vertebrate and mammalian species, although the *Tiki1* gene had been lost in Rodentia. Interestingly the conserved TIKI domain can be traced to prokaryotic TraB proteins, which are negative regulators of sex pheromone signaling in bacteria (Bazan *et al.*, 2013, Sanchez-Pulido and Ponting, 2013). We note that a putative gene product named TRABD represents the third human protein with a distantly related TraB domain. However TRABD shares little sequence similarity with TIKI1 and TIKI2 and is a predicted cytosolic protein, making it unlikely to participate in Wnt cleavage. We note for clarity that several databases identify TIKI1 as TRABD2A and TIKI2 as TRABD2B.

### Tiki expression in the Organizer in chick, rabbit, and mouse embryos

In amphibian *Xenopus laevis* embryos *Tiki1* expression is restricted to the dorsal side of the embryo during gastrula and neurula stages (Zhang *et al.*, 2012). Specifically, *Tiki1* is expressed exclusively in the Organizer at the gastrula stage and becomes restricted afterwards to the prechordal mesoderm and endomesoderm that constitute the “head organizer” (Zhang *et al.*, 2012). The expression pattern of *Tiki1* is similar to that of *Dkk1* in frogs, and like *Dkk1*, *Tiki1* is required for anterior patterning and head formation (Zhang *et al.*, 2012). The anterior deficiency phenotype as a result of *Tiki1* depletion in *Xenopus* embryos is fully rescued by *Dkk1* mRNA injection (Zhang *et al.*, 2012). These findings support the model that inhibition of Wnt signaling by the organizer, through a combination of different molecular strategies, is essential for vertebrate anterior development.

Similar, yet distinct, expression patterns of *Tiki1* and *Tiki2* in the organizer region in chick, rabbit, and mouse embryos are generally consistent with the above model. Although *Tiki1* expression is excluded from the Organizer tissues in the chick, *Tiki2* expression is detected in the Organizer in Hensen’s node and the forebrain organizer, the prechordal plate. These results suggest that *Tiki2*, but not *Tiki1*, has a role in head and forebrain patterning in chick (Table 1). In rabbit embryos, whose early embryogenesis resembles that of humans, *Tiki1* expression is restricted to cells of the hypoblast layer underlying Hensen’s node and its periphery in the earliest stages of development. Subsequently rabbit *Tiki1* expression is detected in the prechordal plate region and in endodermal tissue. Similarly, rabbit *Tiki2* is also expressed in the prechordal plate. These results suggest participation of both *Tiki1* and *Tiki2* in the anterior patterning in rabbits (Table 1).

Mice and other rodents have unique early embryogenesis among mammals in that they develop as an egg cylinder rather than a disc (Arnell and Tam, 2012, Solnica-Krezel and Sepich, 2012, Viebahn, 1999). Somewhat unexpectedly, mice and other rodents appear to have lost the *Tiki1* gene. Whether *Tiki1* loss is related to the unique embryogenesis of rodents is unclear. Nonetheless, mouse *Tiki2* expression is detected in the anterior neural fold and PcP, as observed in chick and rabbit embryos, suggesting a potential role of *Tiki2* in anterior patterning in mice (Table 1).

### **Tiki expression in other aspects of chick, rabbit, and mouse embryogenesis**

*Tiki1* showed a dynamic expression pattern during early chick development with gaps in its expression in some stages of development as seen in E2, showing specific tissue expression from E2.5 onwards, such as dorsal neural tube and maxillary bud (Fig 2). *Tiki2* was present during somitogenesis, in the presomitic and somitic mesoderm in rabbit, however *Tiki2* was not found in the somitic mesoderm in chick embryos. *Tiki2* expression was also detected in mouse as a dorsal stripe along the AP axis, similar to the expression pattern found for *Tiki1* in chick embryos. In chick embryos, the expression of *Tiki2* in the early endoderm and in the AIP is persistent at the early stages of development while the embryo was flattened in a disc shape. On the other hand, rabbit embryos showed an early endoderm expression of *Tiki1*, that became expressed in the PcP and flanking the notochordal plate. Earliest stages expression of *Tiki2* can be seen in all the three germ layers in rabbit embryos. *Tiki2* is expressed in the developing heart of mouse and chick, but not in rabbit which expressed *Tiki1* in the heart anlage (Table 1). These expression patterns suggest that in rabbits, *Tiki1* might be important for early steps of heart induction, while in the mouse and chick, *Tiki2* expression may be important during the heart looping and for cardiac chamber specification. Inhibition of Wnt signaling is critical for heart induction (Marvin *et al.*, 2001). Moreover, it has been reported that signaling pathways involved in the anteroposterior patterning of the neural ectoderm also influence the cardiac chamber formation (Xavier-Neto *et al.*, 2001).

### **Variations of a common theme: distinct Wnt antagonists in embryonic patterning**

Several families of Wnt inhibitors exist in vertebrates with different modes of action for the fine tuning of the Wnt pathway (Cruciat and Niehrs, 2013). Such exquisite control is essential for many development processes such as AP patterning. In fact most known families of Wnt antagonists were discovered in the study of the Organizer function in AP patterning in *Xenopus*, including sFRPs, Cerberus, Dkk1, and *Tiki1*. The expression and requirement of Dkk1, which binds to and inhibits the Wnt coreceptor LRP6 (Bafico *et al.*, 2001, Glinka *et al.*, 1998, Mao *et al.*, 2001, Semenov *et al.*, 2001), is shared among frogs, zebrafish, and mice (De Robertis and Kuroda, 2004, Niehrs, 2004), supporting the notion that the main role of the head organizer is to antagonize Wnt signaling, which instructs posterior patterning (Petersen and Reddien, 2009). However, there seem to be evolutionary variations of this common theme among vertebrates in terms of the usage and molecular nature of other Wnt antagonists. For example, Cerberus in *Xenopus* is a multi-valent inhibitor of Wnt, BMP and Nodal pathways and is involved in head development (Belo *et al.*, 2009). Loss of function of *Cerberus* through morpholino antisense oligo depletion inhibits head formation (Kuroda *et al.*, 2004), while in mouse Cerberus-like does not possess Wnt antagonist activity and its deletion does not affect head formation (Belo *et al.*, 2009). In

a similar vein, while we found that Tiki1 in *Xenopus* is required for head development (Zhang *et al.*, 2012), rodents including mice appear to have lost the Tiki1 gene and thus are different from other vertebrates/mammals examined, which have both Tiki1 and Tiki2 genes. Furthermore, while chick and rabbit embryos exhibit Organizer Tiki expression like *Xenopus* embryos, Tiki2, but not Tiki1, shows Organizer-specific expression in chick while Tiki1 and Tiki2 share co-expression in the Organizer in rabbits. These variations may reflect some stochastic events during evolution or unique aspects of early embryogenesis in different vertebrates while preserving the fundamental function of the head organizer in antagonizing Wnt signaling.

### Tiki expression in adult tissues in human and mice

In human the TIKI1/TRABD2A mRNA is detected in adult tissues including the small intestine, colon, lung and ovary while TIKI2/TRABD2B is detected in the kidney, heart, adipose tissue, and gallbladder (www.proteinatlas.org). This tissue distribution of human TIKI2 is in agreement to that previously reported for mouse Tiki2 (Zhang, 2012), whose expression in fat is regulated by diet (Zhang, 2012). Thus *TIKI* genes may have dynamic expression in adult tissues under physiological and pathological conditions. Indeed database search shows that expression of TIKI1 and/or TIKI2 is altered in a variety of cancers and disease tissues (Supplemental Table 1). Indeed consistent with our findings that TIKI2 expression is downregulated in osteosarcoma (Supplemental Table 1), forced expression of TIKI2 inhibits osteosarcoma cell growth and cancerous behaviors in culture and in mouse xenografts (Li *et al.*, 2014b). Therefore like DKK1 and SOST, which is a Wnt antagonist mutated in skeletal disease sclerosteosis (Baron and Kneissel, 2013), TIKI1 and TIKI2 may be involved in human pathogenesis.

## Materials and Methods

### Plasmid construction of Tiki probes

Using available genomic and EST sequences, primers were made to amplify full-length Tiki cDNAs (or as much as possible) for chicken (*Gallus gallus*), rabbit (*Oryctolagus cuniculus*) and mouse (*Mus musculus*) Tiki genes. Full-length vertebrate Tiki proteins are encoded by seven exons, however the sequence of the first and/or the last exon was not always available due to promoter high GC content or low sequence homology. A chicken Tiki1 partial cDNA probe (1073bp, ChT1exon2F AGCTGAACTCCTTCCTGTGG and ChT1exon6R CTCCTCAGGGTATGCAACG) and a chicken Tiki2 full length cDNA probe (1614bp, ChT2exon1F GGGATGCAACTCAACCTGAC and ChT2exon7R CTCTATTCCAAGGGATTGCAC) were generated using RT-PCR from RNAs of stage HH21 White Leghorn chick embryos and TOPO cloned into the pCRII-TOPO vector (Invitrogen). Using similar methods, a full-length Rabbit Tiki1 cDNA (1553 bp, RaT1exon1F CCGTTGTCGGGAGGATG and RaT1exon7R GCCTGGCTCCTCAGTCATTC) and a partial Rabbit Tiki2 cDNA (1471 bp, RaT2exon2F CCTGAACTCCTTCCTGTGGA and RaT2exon7R GTGGCCGAGGTCAGGAG) were amplified via RT-PCR of RNAs from adult New Zealand White rabbit intestine and kidney, respectively, and TOPO cloned into the pCRII-TOPO vector. A full-length mouse Tiki2 cDNA (1517 bp MoT2exon1F TAACTCGAGCCACCATGCACGCCGCCCTGGC and



MoT2<sup>exon7R</sup> TAATCTAGATCAGGAAGGCCCAAGGCTGTG) was amplified via RT-PCR of RNAs from cultured mouse L cells (ATCC CRL-2648) and cloned into the pCS2+ expression vector using XhoI/XbaI sites. Antisense probes were made using SP6 or T7 polymerase from linearized plasmid vectors. Mouse Tiki2 cDNA was also used to make a <sup>32</sup>P random labeled probe (Rediprime II, Amersham) for detecting Tiki2 transcripts from a Clontech northern blot containing total RNA from adult mouse tissues. The northern blot was reprobed with a Gapdh probe (448 bp, GapdhF GCCAAAAGGGTCATCATCTC and GapdhR CCTGCTTCACCACTTCTTG) as a control.

### Chick, rabbit and mouse embryos

Fertilized chick eggs were incubated at 37°C and staged according to Hamburger and Hamilton (1951). Mated female rabbits (New Zealand White) were purchased from a commercial breeder (ZIKA, Germany) and sacrificed according to German animal law. Embryos from timed pregnancies were dissected from uteri in PBS at room temperature and fixed for in situ hybridization in 4% PFA at 4°C overnight. Embryos were staged according to Blum et al. (2007). Mouse embryos were obtained from timed pregnant C57BL/6J females as previously described (Adamska *et al.*, 2003). Noon of the day when the vaginal plug was found was considered to be E0.5.

### Whole mount in situ hybridization

Whole-mount in situ hybridization using digoxigenin-labeled riboprobes was performed following standard protocols or as described in (Brito *et al.*, 2008). Chick embryos from stage HH4 to HH20-21 were fixed in 4% formaldehyde at 4°C overnight, and then dehydrated in a methanol series.

After *in situ* hybridization, chick embryos were dehydrated in ethanol series, cleared in butanol and embedded in paraffin wax. Cross sections at 10 or 20 µm were performed to show internal structures. Rabbit embryo histology was performed as described (Fischer *et al.*, 2002).

### Phylogeny analysis and protein sequences

The Tiki genes tree was clustered using a Neighbor-Joining phylogenetic method (Mega5 software). The phylogenetic analysis of TIKI proteins was tested using a bootstrap method and resampled at least 1000 times. The result is a percentage of times that a particular grouping was on each side of a branch without concerning the subgrouping. TIKI protein sequences were derived from full-length NCBI accessions and genomic sequences. Chicken and Rabbit Tiki1/2 ESTs were independently cloned for use as in situ probes. Human [*Homo sapiens*] TIKI1: AFN02881.1 and NP\_001263982.1, TIKI2: AFN02882.1 and NP\_001181915.1; Macaque [*Macaca mulatta*] Tiki1: EHH22275.1, Tiki2: XP\_001101255.2; Rabbit [*Oryctolagus cuniculus*] Tiki1: based on genomic sequence and XP\_002709727.1 (missing exon 3), Tiki2: based on genomic sequence for exons2-7, exon1 was inferred from Human/Mouse; Mouse [*Mus musculus*] Tiki2: NP\_001079018.1; Rat [*Rattus norvegicus*] Tiki2: based on genomic sequence and XP\_001072500.2; Guinea Pig [*Cavia porcellus*] Tiki2: based on genomic sequence and XP\_003461517.1; Squirrel [*Ictidomys tridecemlineatus*] Tiki2: based on genomic sequence and XP\_005318067.1;

Chicken [*Gallus gallus*] Tiki1: XP\_003643097.2, Tiki2: XP\_422458.4; *Xenopus* [*Xenopus* (*Silurana*) *tropicalis*] Tiki1: AFN02883.1 and NP\_001269420.1, Tiki2: AFN02884.1 and P0DJQ9.1; Cnidaria [*Nematostella vectensis*] Tiki: AFN02888.1 and A7RX69.2. The identity between species was determined by GeneCode software.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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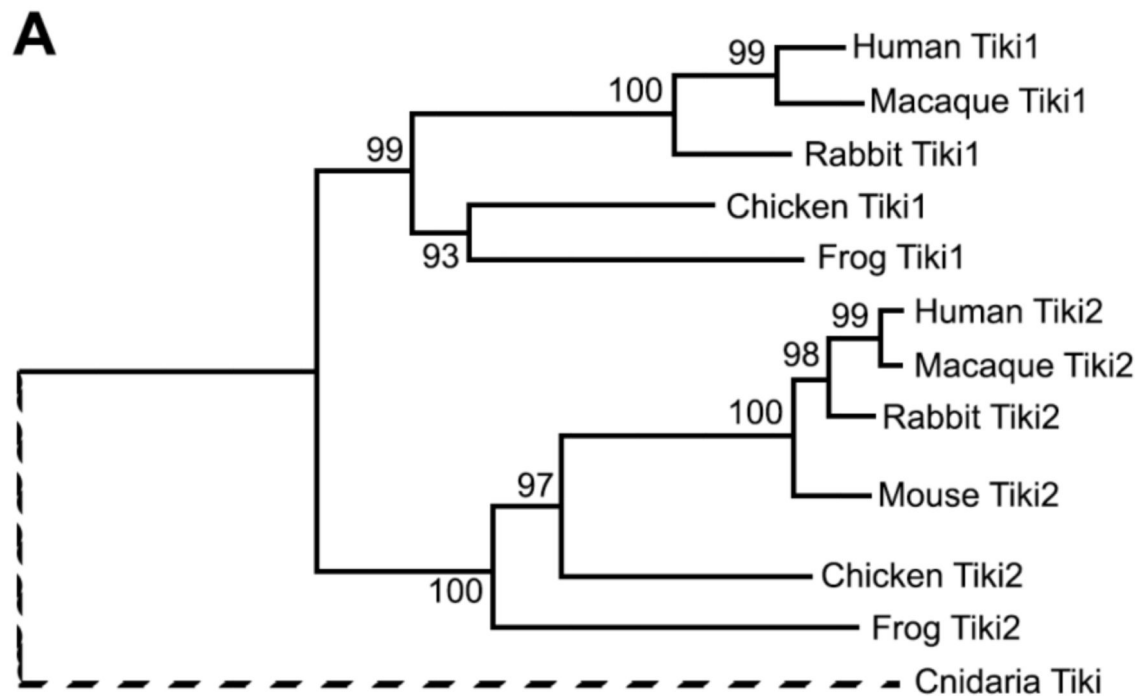
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**B**

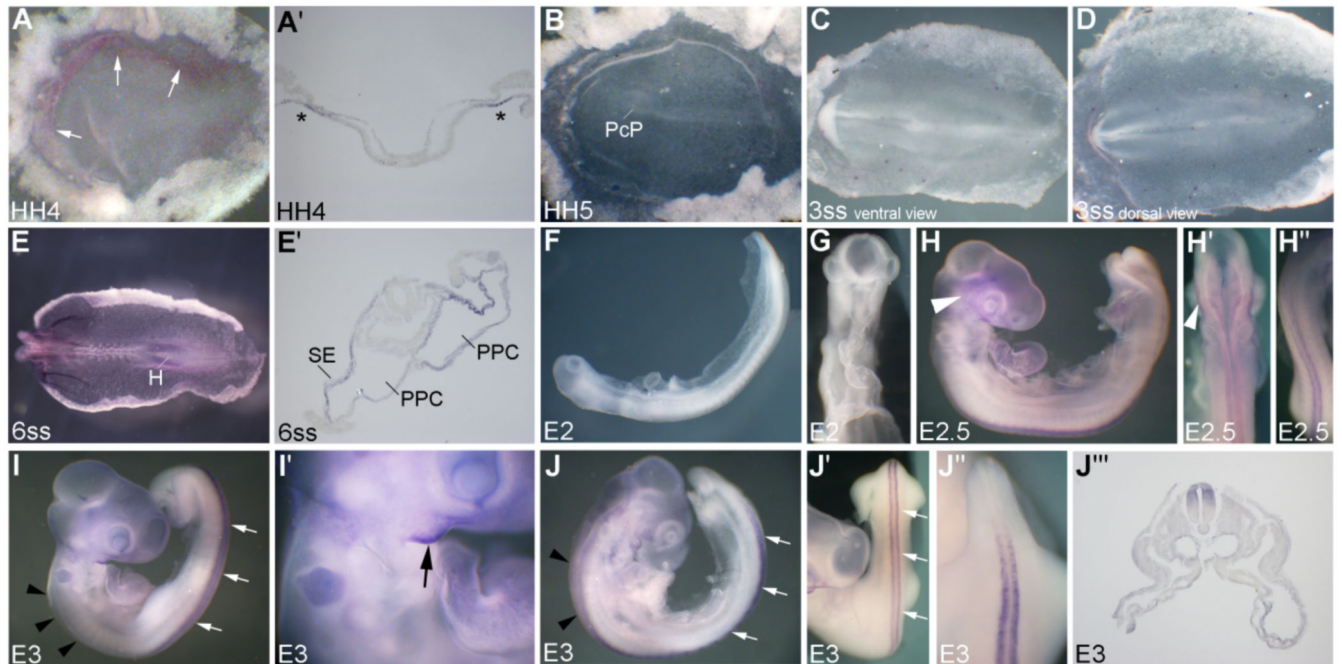
	Human Tiki1	Macaque Tiki1	Rabbit Tiki1	Chicken Tiki1	Xenopus Tiki1
Human Tiki1	***	94.1	86.7	68.3	65.5
Macaque Tiki1		***	86.3	67.9	64.5
Rabbit Tiki1			***	69.3	66.1
Chicken Tiki1				***	74.5
Xenopus Tiki1					***

	Human Tiki2	Macaque Tiki2	Rabbit Tiki2	Chicken Tiki2	Xenopus Tiki2
Human Tiki2	***	97.9	92.2	72.3	65.7
Macaque Tiki2		***	92.1	72.1	65.3
Rabbit Tiki2			***	73.0	67.4
Chicken Tiki2				***	70.5
Xenopus Tiki2					***

**Figure 1. Evolutionary comparisons of vertebrate Tiki proteins**

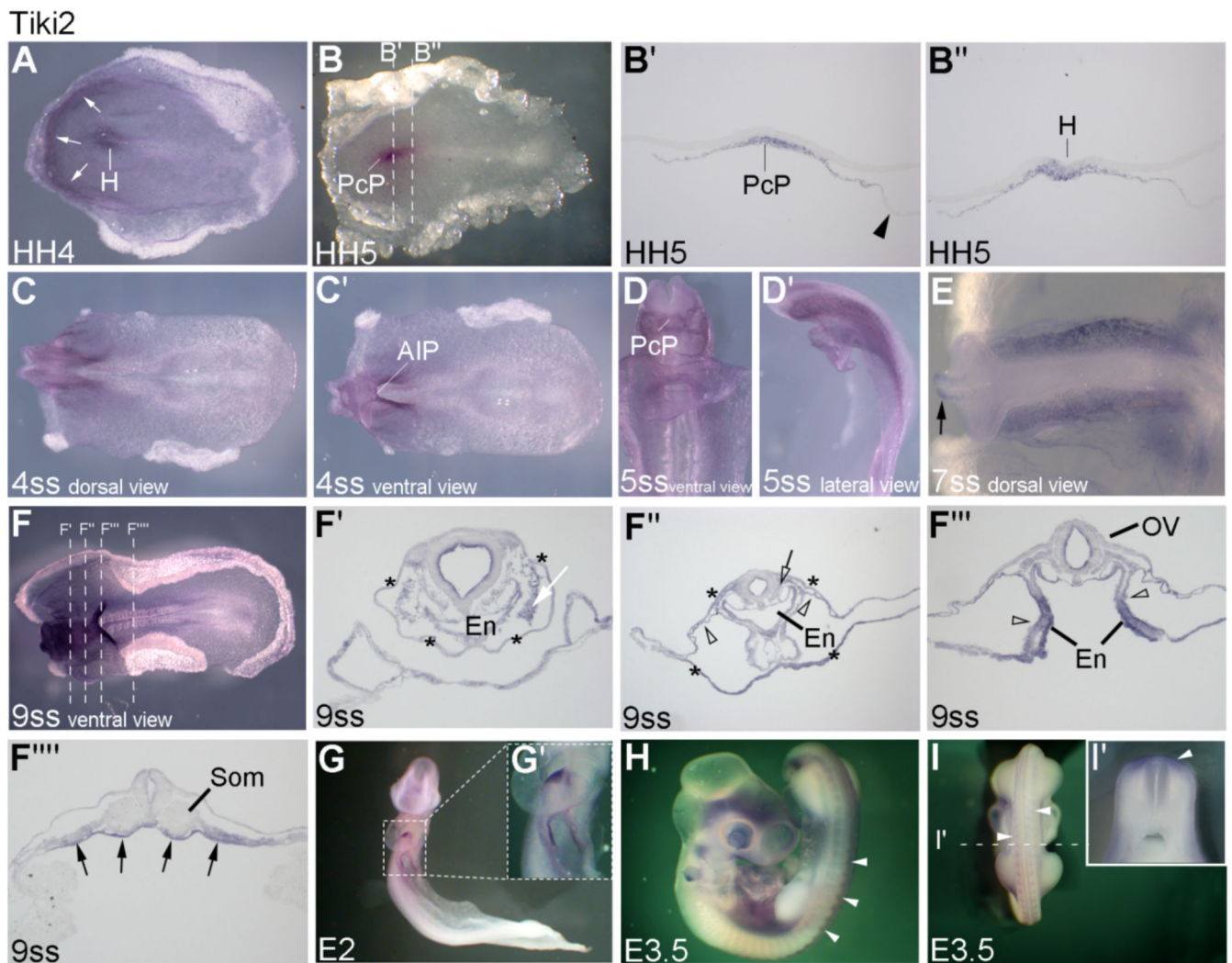
(A) The phylogenetic tree showing homology among Tiki proteins from Human, Macaque, Mouse, Rabbit, Chicken and *Xenopus* based on Neighbor-Joining phylogenetic methods. Distances in the cladogram are derived from amino acid substitutions. The full length protein sequences are shown in Fig. S1. Tiki1 is absent from the mouse and all sequenced rodents (see Fig. S2B). Cnidarian Tiki protein is used as an out-group. (B) Orthologous comparisons of Tiki1 and Tiki2 proteins in percent identity using ClustalW alignment.

## Tiki1



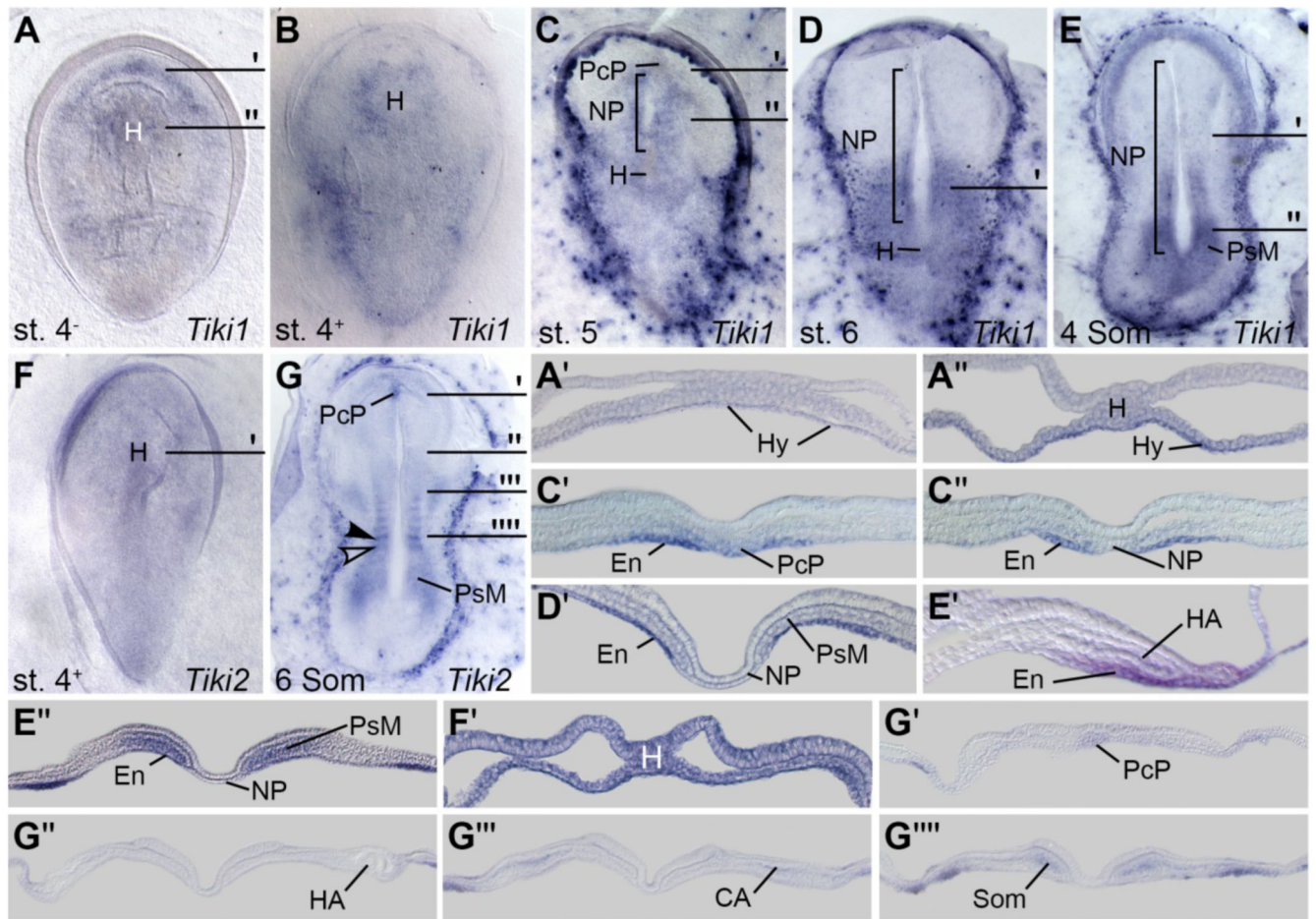
**Figure 2. In situ hybridization analysis of *Tiki1* during chick embryonic development**

*Tiki1* transcripts were analyzed by in situ hybridization in whole mount or cross sections at stage HH4 (**A**, white arrows indicate expression in the anterior, lateral and posterior area pellucida, and **A'**, asterisks mark expression in the epiblast and hypoblast), HH5 (**B**), the 3-somite stage (ss) (**C**, ventral view, and **D**, dorsal view), the 6ss (**E** and **E'**), E2 (**F**, lateral view, and **G**, head ventral view), E2.5 (**H**, **H'**, and **H''**; the arrowhead indicates lateral forehead ectoderm), E3 (**I**, **I'**, **J**, **J'**, and **J''**; the black arrow and arrowheads indicate the maxilar bud and anterior neural tube, respectively, and the white arrows mark the posterior neural tube). A posterior cross section at E3 was shown (**J'''**). Abbreviations: PcP, prechordal plate, H, Hensen's node, SE, surface ectoderm, and PPC, pleural-peritoneal cavity.



### Figure 3. In situ hybridization analysis of *Tiki2* during chick embryonic development

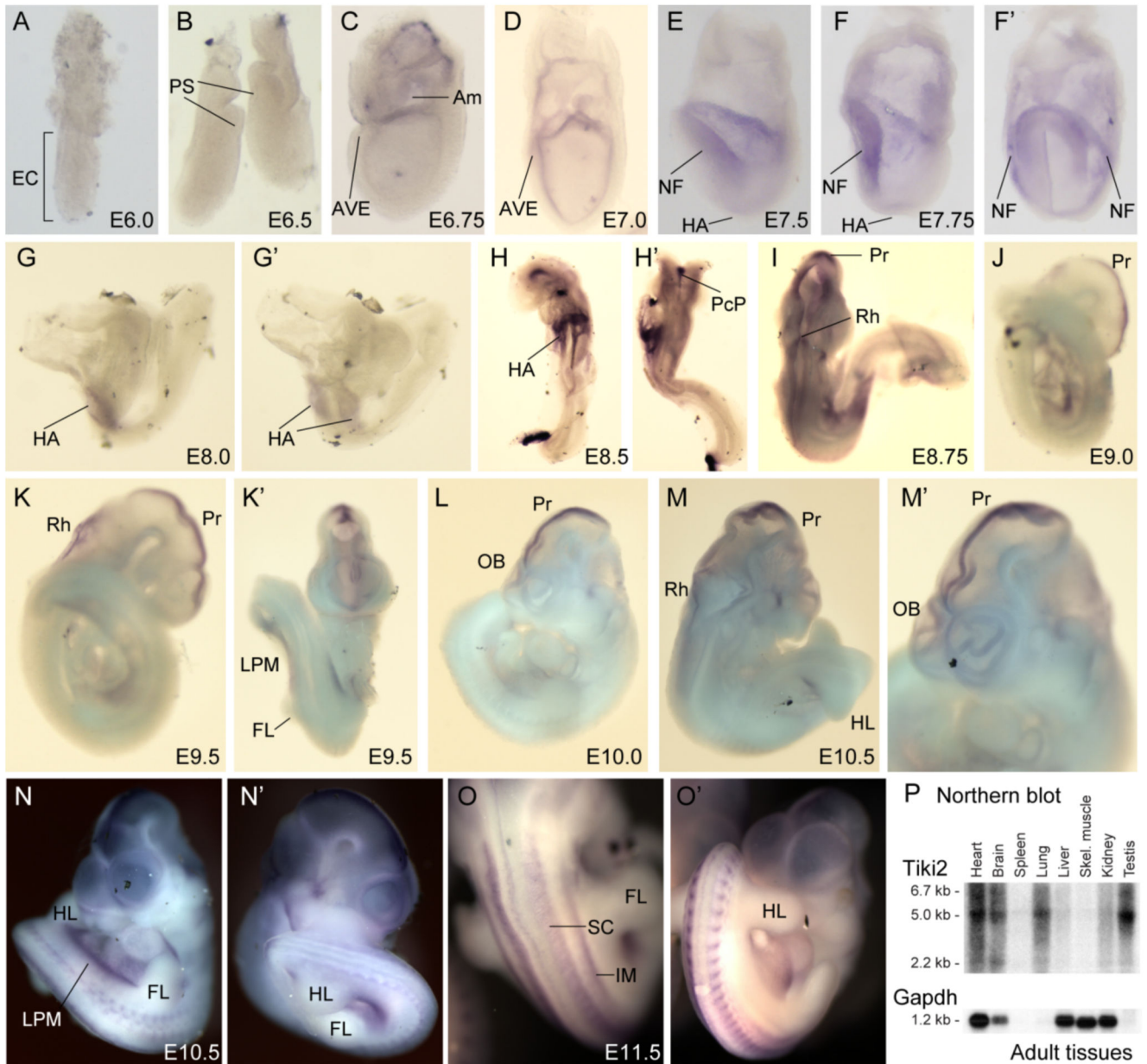
*Tiki2* transcripts were analyzed by in situ hybridization in whole mount or cross sections at stages HH4 (A, arrows indicate the germinal crescent), HH5 (B), HH5 sectioning at the level of prechordal plate (B', the black arrowhead indicates the lateral endoderm) and Hensen's node (B''), the 4ss (C, dorsal view, and C', ventral view), the 5ss (D, ventral view, and D', lateral view), the 7ss (E, black arrow: neural ectodermal ridge), the 9ss (E, ventral view, white dashed lines indicate the level of the cross sections), the 9ss cross sections (F', F'', F''', and F'''), white arrow: the head mesenchyme; asterisk: the lateral/ventral ectoderm; empty arrow: paraxial mesoderm; empty arrowhead: the lateral plate mesoderm; black arrow: dorsal and lateral endoderm), E2 (G and G'), E3.5 (H, lateral view, I, dorsal view, I', cross section at the level of the trunk indicated by the dashed lines, white arrowheads indicate expression in the ectoderm dorsal and lateral to the neural tube at the trunk). Abbreviations: H, Hensen's node, PcP, prechordal plate, AIP, anterior intestinal portal, En, endoderm, Som, somite, and OV, the otic vesicle.



**Figure 4. In situ hybridization analysis of *Tiki1* and *Tiki2* during early rabbit embryonic development**

Whole-mount *in situ* hybridization of rabbit embryos of defined stages (st.) using antisense probes specific for rabbit *Tiki1* (A - E) and *Tiki2* (F, G). Rabbit embryos were staged according to Blum et al. (2007). Specimens are shown in ventral views with anterior to the top. Histological sections (A' and C' - G'') in A and C - G are shown dorsal side to the top. (A, A', A'') Expression in hypoblast (Hy) cells in an anterior crescent-shaped domain and underlying Hensen's node (H) in a st. 4<sup>-</sup> embryo. (B) Expression around the area of Hensen's node in a st. 4<sup>+</sup> embryo. (C, C', C'') Rostro-caudally-expanding expression in the endoderm (En), expression in the prechordal plate (PcP) bordering the developing notochordal plate (NP) in a st. 5 embryo. (D, D') Expression in endodermal cells of the posterior half of a st. 6 embryo. The presomitic mesoderm (PsM) was devoid of *Tiki1* transcripts. (E, E', E'') *Tiki1* expression emerged in the endoderm underlying the caudal aspect of the heart anlage (HA), in the presomitic mesoderm and underlying endoderm in a 4 somite (Som) stage embryo. (F, F') *Tiki2* was expressed ubiquitously before somitogenesis. (G, G', G'', G''', G''') Expression of *Tiki2* in the prechordal plate, presomitic mesoderm and the compacting (empty arrowhead) and compacted (arrowhead) somites. Note the absence of expression in the heart anlage and emerging expression in the coelom anlage (CA).








### Figure 5. Expression of *Tiki2* during mouse embryonic development

(A-O) Whole-mount in situ hybridization of mouse embryos of defined stages using antisense probes specific for mouse *Tiki2*. (A and B) *Tiki2* expression is absent in the egg cylinder (EC) and primitive streak (PS) stages. (C) *Tiki2* was first detected in the extraembryonic tissues after closure of the amnion (Am) and was notably absent from the anterior visceral endoderm (AVE) region. (D) *Tiki2* is diffusely expressed in the embryo proper at E7.0. (E and F) Distinct *Tiki2* expression domains in the neural folds (NF) and heart anlage (HA) were found at E7.5 and E7.75, shown with the anterior facing left or (F') as an anterior view of the embryo. (G, G', H and H') *Tiki2* is expressed in the right and left fields of the heart anlage at E8.0 and E8.5, and was found in the prechordal plate (PcP) at E8.5 as the embryo begins to turn. (I) Neural fold *Tiki2* expression was detected during

neural tube closure along the prosencephalon (Pr) and rhombencephalon (Rh) and (**J-N**) was prominently expressed along the mid sagittal prosencephalon from E9.0-E10.5. (**L-M'**) Rostral prosencephalon expression continued into the presumptive olfactory bulb (OB) region. (**J-N'**) *Tiki2* was not detected in the fore limb (FL) or hind limb (HL) buds, but was prominent in lateral plate mesoderm (LPM) between the limb buds. (**O** and **O'**) *Tiki2* expression became more elaborate at E11.5 and was detected in the developing spinal cord (SC) and intermediate mesoderm (IM). Littermate embryos from the early stages were probed with *Fgf8* in parallel to control for RNA integrity (data not shown). (**P**) Northern blotting of total RNAs of adult mouse heart, brain, spleen, lung, skeletal muscle, kidney and testes (Clontech) was performed for the mouse *Tiki2* and *Gapdh*.

**Table 1**

Summary of *Tiki1* and *Tiki2* expression during chick, rabbit and mouse early development.

			
<i>Tiki1</i>	Hypoblast/epiblast, surface ectoderm, LPM, maxillary bud, dorsal spinal cord.	Hypoblast/endoderm AMC, Hensen's node, PcP, PsM, somites, HA.	—
<i>Tiki2</i>	Hensen's node, PcP, AIP, head mesenchyme, endoderm, primitive atrium.	PcP, PsM, somites.	Anterior neural fold, heart anlagen, LPM, IM, PcP, surface ectoderm, dorsal spinal cord.

Anterior intestinal portal (AIP), Anterior marginal crescent (AMC), Presomitic mesoderm (PsM), heart anlage (HA), Prechordal plate (PcP), Intermediate Mesoderm (IM), Lateral Plate Mesoderm (LPM).