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Review

Tribbles:
novel regulators of cell function; evolutionary aspects

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Abstract. Identification of rate-limiting steps or components of intracellular second messenger systems holds promise to effectively interfere with these pathways under pathological conditions. The emerging literature on a recently identified family of signalling regulator proteins, called tribbles gives interesting clues for how these proteins seem to link several 'independent' signal processing systems together. Via their unique way of action, tribbles co-ordinate the activation and suppression of the various interacting signalling pathways and therefore appear to be key in determining cell fate while responding to environmental challenges. This review summarises our current understanding of tribbles function and also provides an evolutionary perspective on the various tribbles genes.

Keywords. Signal transduction, tribbles, MAPK, Akt, ATF4, regulation of signalling, scaffolds.

Introduction

A fundamental feature of all life forms is the ability to respond to changes in the extracellular environment. In order to perform this task efficiently, specialised intracellular signal processing (second messenger) systems have evolved, which transmit the signals, generated by cell surface sensors (receptors). The co-ordinated action of these signal-processing machineries ultimately results in the appropriate cellular response via changes, for instance, in gene expression or activation of proteins involved in cell cycle progression or apoptosis. However, the highly optimised spatio/temporal regulation of the activity of various second messenger systems is key to the maintenance of homeostasis. Therefore, a large diversity of control mechanisms are in place to warrant optimal performance. These include non-catalytic components of the signalling pathways, which play a key role in the localisation and assembly of active signalling complexes, ultimately regulating the activation of the pathway. Scaffold proteins have been proposed to be of key importance in regulating the output of signalling systems [1, 2]. Mathematical modelling of scaffold function suggest that they can confer kinetic regulatory properties on MAPK activation [1, 2]. Depending on their concentration, scaffolds may be able to potentiate or inhibit MAPK function [1].

A number of mammalian MAPK scaffolds have been reported in recent years (for a recent review see [3]). Most of these proteins have been shown to associate with one or more MAPKs, MAPKKs or MAPKKKs, thus assembling functional signalling complexes. However, it is clear that there may be novel proteins with scaffold-like regulatory function, which remain to be identified.

An emerging literature on a recently identified protein family, called tribbles suggest that these proteins may well represent 'bottle necks' with a scaffold-like regulatory function for a number of signalling pathways and thus play a role in embryonic development and the devel-

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opment of human diseases, such as cancer, autoimmune disease and diabetes. In this review, we set out to summarise the current literature on tribbles and also discuss the evolution of this protein family, in the light of currently available genome/expressed sequence tag (EST) sequencing data.

Tribbles in invertebrates

A number of studies were published in 2000, describing a novel regulator of *Drosophila melanogaster string* in morphogenesis. *String* is the fly orthologue of mammalian *cdc 25*, a phosphatase, which plays a key role in regulating cell cycle progression beyond G2 by de-phosphorylating, and thus activating, the cyclin-dependent kinase, *cdk 1*. In embryonic development, expression of *string* is usually accompanied by the progression of mitosis. However, during morphogenesis, high levels of *string* are expressed in the gastrulating mesoderm anlage and yet, the migrating cells do not divide. To solve this long-known puzzle, a number of groups have performed genetic screens with the aim to identify inhibitors of *string*. Großhans and Wieschaus carried out a genome-wide survey for genes that are required for delayed mitosis during the formation of the ventral furrow. They identified two genes with such function, *tribbles* [4] and *frühstart* [5]. Genetic analysis suggested a distinct role for these two genes in a common pathway. These authors show that overexpression of *trb* inhibits mitosis by microinjecting a large amount of *trb* mRNA into the posterior half of the embryos. However, they also show that *trb* expression itself is not sufficient to block mitosis, rather it requires an unknown, upstream, activating signal.

In a parallel study, published by Seher and Leptin, a screen was performed for genes whose disruption results in a deficient gastrulation [6]. They show that the primordial cells in tribbles$^{-/-}$ mutants exert a low level of mitotic activity, not seen in wild-type embryos. In addition, a few cells in the mutant mesoderm will also undergo an extra cell division. However, in neither situation is the trb$^{-/-}$ phenotype uniform within the cell population involved in the morphogenic movements. Further, they were unable to block mitosis in other cells by supplying high levels of tribbles mRNA. Again, these findings imply that trb$^{-/-}$ may not be the only protein required for blocking mitosis during morphogenesis.

Roth *et al.* [7] provide a molecular model for trb function. They show that *slbo*, a fly homologue of mammalian C/EBP transcription factor, levels are regulated by tribbles, possibly in a ubiquitin-dependent manner.

A further paper, published in parallel to the previous studies investigates *trb* function in a different developmental setting, oogenesis. Mata *et al*. [8] show that *trb* has a regulatory role in the highly specialised sets of cell divisions during oogenesis. This process starts with an asymmetric division of a germline stem cell followed by four synchronous divisions of the daughter cystoblast that gives rise to 16 cystocytes. One of these will eventually become the oocyte and initiate meiosis, while the others will develop as nurse cells. Mata *et al*. [8] showed that trb mutant embryos undergo an extra cycle of cell division, resulting in 32 cystocytes. Wing development in flies is also affected by *tribbles*. When *trb* is overexpressed in the posterior compartment of the wing imaginal disk, the wing is made up of fewer but larger cells, compared with controls. (This enlarged phenotype was also seen in HeLa cells, when human trb-3 was overexpressed [9].) Flow cytometry analysis suggested that *trb* expressing cells were mainly in G2/M phase. Further experiments confirmed that, similar to the phenotype seen during gastrulation [4, 6], *tribbles* down-regulates *string*. Co-transfection experiments, using epitope-tagged string and *twine* (another Cdc25 homologue in fly) demonstrated that *tribbles* reduce the protein levels of these cell cycle regulating phosphatases.

In summary, *Drosophila tribbles* have been shown to regulate embryonic development at several stages. Two models have been proposed to explain *trb* action. It has been suggested that *trb* expression regulates C/EBP and Cdc25 protein levels by mediating their degradation. However, it is clear from the above studies that *trb* only exerts this activity in specialised settings, suggesting that *trb* activity itself may also be regulated by upstream signals in a fashion, which is necessary for its downstream action. Alternatively, it is also possible that *trb* acts as part of a larger protein complex and the expression/activity of other, yet uncharacterised components are crucial for trb-mediated regulation of embryonic development.

Tribbles gene family in vertebrates

Xenopus tribbles

The biological role of frog trb-2 in development has been investigated [10]. It was shown that trb-2 was maternally expressed in the oocytes. Similarly to mammalian counterparts (see below), xtrb-2 mainly localised in the cytoplasm. However, re-distribution of the protein was observed during cell division. In contrast to the *Drosophila* phenotype, microinjection of the fertilised *Xenopus* embryos with an antisense morpholino against trb-2 led to delayed (and not accelerated) cell division. Defects in the development of the eye and the nervous system were also seen.

One explanation for this stark contrast between the fly and frog phenotypes could be that the *Drosophila* eggs were trb$^{-/-}$, while the *Xenopus* oocytes contained some maternally expressed trb-2 protein. Another important difference between the two model organisms is that, while flies

have a single *trb* gene, *Xenopus* have both trb-1 and trb-2 (see below). It is possible that eliminating just one of the two *trb* proteins leads to a different phenotype compared with a complete $trb^{-/-}$ embryo.

Mammalian tribbles

Tribbles-1. Human tribbles-1 was first identified (named c8fw) as a homologue of trb-2 (originally called c5fw) by Wilkin *et al*. [11], based on a partial cDNA sequence. The human gene is located on chromosome 8 at q24.13. trb-1 has also been reported as a protein interacting with 12-lipoxygenase (12-LOX) in a yeast two-hybrid screen [12]. However, the biological relevance of this interaction is still unknown as there has been no independent confirmation and further characterisation of trb-1/12-LOX binding. Our group have reported recently the identification of human tribbles-1 as a novel regulator of AP-1 activation in a screen [9], which is designed to rapidly annotate signalling network components based on their function [13–15]. Further characterisation of trb-1 function revealed that this protein (similarly to trb-3) binds to the 'middle layer' of kinases in the MAPK network, the MAPKKs [16]. Specifically, trb-1 interacts with MEK1 and MKK4 in HeLa cells. These interactions, in turn, regulate the activity of MAPKKs and also modulate the protein expression level of trb-1 (and trb-3) itself [16], presumably via influencing trb turnover/stability. We have found recently that trb-1 expression is regulated by inflammatory stimulation; mRNA levels are up-regulated in response to the stimulus in a variety of cell types [17]. We have reported previously that trb-1 is a nuclear protein and that the N-terminal, proline-rich domain is necessary for this localisation [9]. An interesting clue to the biological relevance of varying trb-1 expression levels comes from a recent publication by Johansson's group [18]. They investigated the consequence of the amplification of a 4.3-Mb region on chromosome 8 in myeloid malignancies. It has been suggested that patients with such amplifications have a poorer prognosis. Since c-*myc*, a major oncogene, is harboured by these amplified regions, it is widely believed that an increase in *myc* gene copy number leads to elevated protein levels potentiating development of the malignant phenotype. However, Storlazzi *et al*. [18] have demonstrated that of the eight known genes encoded by the amplified region of chromosome 8, only trb-1 expression is up-regulated. Putting this observation together with the *Drosophila* data (see above), where it has been shown that $trb^{+/-}$ flies have a characteristic phenotype, it is tempting to speculate that alterations in trb-1 gene dose may have important functional consequences in certain situations. It is worth noting, however, that the experimental data presented on the human malignancies [18] was based on only a small set of samples. It will therefore be very interesting to see whether analysis of larger cohorts lead to the same conclusion.

Tribbles-2. Amongst the family of mammalian tribbles, the least is known about the molecular function of trb-2. The human gene is located on chromosome 2, at p24.3. The first identification came from Wilkin *et al*. [19], who described a number of genes, for which expression is up-regulated by mitogens in dog thyroid cells. They subsequently showed that the protein is phosphorylated on multiple sites [11]. However, the sites of phosphorylation and the kinase(s) responsible are yet to be identified. In addition, the biological relevance of trb phosphorylation is currently unknown. It has been shown by a number of groups that trb-2 is mainly localized in the cytoplasm of a variety of cell types [9, 11, 20] and that it is a labile protein [11].

The first human trb-2 cDNA sequence, expressed in osteoblasts, was deposited in GenBank under the name of GS3955 by Ohno *et al.*, in 1998. A number of studies describe trb-2 as a differentially expressed gene in various situations. It has been shown that this trb is abundantly expressed in kidney mesenchymal cells [21]. Our group have found recently that the regulation of expression of this gene (similarly to other tribbles) is cell-type specific [17]. While trb-2 expression is up-regulated by 6 h in THP-1 cells when stimulated by IL-1β, mRNA levels of the same gene decrease sharply (and transiently) in response to an identical stimulus in synoviocytes. Further, Bisoffi *et al*. [20] have recently shown that trb-2 is strongly (over 40-fold) up-regulated in androgen-independent bone metastatic prostate cancer cells, compared with non-metastatic counterparts.

A recent publication from Li's group [22] demonstrates that trb-2 is a candidate autoantigen in autoimmune uveitis. They used a phage display library to identify autoantigens. A GST-trb-2 fusion protein was then used in an ELISA assay to demonstrate the presence of anti-trb antibodies in uveitis patients. Interestingly, three of the ten patients showed a significant increase in ELISA signal, compared with none of the ten controls measured. Since autoimmune uveitis is a chronic inflammatory disease, these results are in line with the observation that trb-2 expression is regulated by inflammatory cytokines [17].

In summary, mammalian trb-2 is a cytoplasmic, labile protein, the expression of which is regulated by mitogens and during inflammation *in vitro* and *in vivo* and it may also play a role in tumour metastasis.

Tribbles-3. Trb-3 is the best studied member of the mammalian tribbles family. The human gene is located on chromosome 20 at p13-p12.2. Identification of this protein was first reported by two groups in 1999. Mayumi-Matsuda and colleagues [23] described it as a gene that is up-regulated in a neuronal cell line undergoing nerve

growth factor withdrawal induced apoptosis. In a study published in parallel, Klingenspor *et al*. [24] screened for differentially expressed genes in the fatty liver dystrophy (*fld*) mouse. In this study, trb-3 was reported as *Ifld2*, a gene highly induced in new born animals. The first clues on trb-3 function came from several reports published in 2003, describing trb-3 as a regulatory protein for ATF4 [25], p65/RelA [26] and Akt/PKB [27]. In the first study, D. and T. Örd showed that trb-3 interacts with the N-terminal region of ATF 4 in a yeast two-hybrid system. The first 125 residues of the transcription factor have been shown to be necessary for this interaction. trb-3/ATF4 interaction was confirmed in a transient expression/co-precipitation system as well. The functional relevance of this was tested in a reporter assay, confirming that trb-3 has a negative regulatory effect on ATF4-mediated gene activation. Since the ATF4 target genes include CHOP and HO-1, both of which are involved in the regulation of apoptosis, it is plausible to link the modulation of trb-3 expression in apoptotic cells to the co-ordination of programmed cell death. A recent development on this hypothesis comes from Okoha *et al*. [28] who described a feedback mechanism demonstrating that the ATF4-CHOP pathway itself regulates expression of trb-3. Interaction between trb-3 and the N terminus of CHOP was shown. While this interaction had no effect on CHOP turnover, increasing trb-3 levels inhibited CHOP-mediated transcription. Since CHOP and ATF4 co-operate in inducing trb-3 expression, interactions between trb-3, ATF4 and CHOP create a self-regulatory network to fine-tune trb-3 levels and, in turn, will have an impact on trb-3-mediated cell death as well. In line with this is a recent report by D. and T. Örd showing that trb-3 expression is up-regulated in stressful conditions via the ATF4/CHOP pathway [29].

In a study published by Wu *et al*. [26], a yeast two-hybrid screen was performed with the aim of identifying proteins associated with a TNF receptor, BCMA. One of the proteins characterized was trb-3. However, this interaction was not confirmed in mammalian cells. Rather, they report that trb-3 interacts with p65/RelA and inhibits is activation via PKAc phosphorylation. They also show that overexpression of trb-3 sensitises cells to TRAIL induced apoptosis. Similar observations were reported by Huang *et al*. [30]. However, the relationship between the ATF4/CHOP and p65 system is currently unknown. Since ATF4 and CHOP both have been shown to be inducers of apoptosis and p65 generally promotes survival, it is tempting to speculate that trb-3 may be the decision point between these two cellular responses under stress conditions. An interesting recent study supports the potential regulatory role of tribbles-3 under stressful conditions by demonstrating that cellular stress can both up- and down-regulate trb-3 expression [31].

The third line of investigations on trb-3 cellular function centres around the theme of nutrient starvation/glucose metabolism. A report from Montminy's lab [27] described the identification of trb-3 as a binding partner for PKB/Akt in a yeast two-hybrid assay. They showed that trb-3 inhibits Akt activation by preventing its phosphorylation. The proposed mechanism for this effect is a direct trb binding to the activation domain of Akt and therefore masking the Thr^{308} phosphorylation site. To assess the physiological significance of trb-3 on Akt-mediated processes, they show that trb-3 levels are elevated during fasting and that adenovirus-mediated expression of trb-3 in mice leads to alterations in glucose metabolism *in vivo*. A second report from the same group [32] showed that trb-3 is a molecular target for PPAR-α, a protein which is activated by fasting. They propose that regulation of trb-3 levels serve as a major modulatory mechanism in the balance of glucose metabolism. However, a further publication questions the role of trb-3 in this process, based on a number of carefully controlled experiments [33]. Iynedjian showed that adenovirus-mediated overexpression of trb-3 in rat primary hepatocytes has no effect on insulin induced Akt activation. Further, as opposed to glucocorticoid- and forskolin-treated hepatome cells [27], the expression of trb-3 was unaffected in insulin-treated primary liver cells [33]. The basis for the difference in the findings of the two groups is currently unclear. One important difference between the two studies is that, while the effect of overexpressed trb-3 on endogenous Akt was studied by Iynedjian, Du et al. [27] used a system in which both proteins were overexpressed. Also, the cell types studied were different. Since we have shown that trb action is cell-type specific [16] and that the relative level of trb expression compared with its binding partners is critical, both of these differences may be the source of the observed discrepancy. Clearly, further studies are needed to resolve this conflict and clarify the involvement of trb proteins in the modulation of glucose metabolism.

However, some recent indirect evidence further argues for trb-3 as an important molecule in nutrient starvation. Schwarzer *et al*. [34] showed that trb-3 expression is critically dependent on PI3K activity in PC-3 cells. Further, they demonstrated that glucose or amino acid starvation induces trb-3 expression. Interestingly, the activation of trb-3 expression was not dependent of Akt activity. Further, high trb-3 levels did not cause an inhibition of Akt phosphorylation. In contrast to the study published by Wu *et al.* [26], they showed that overexpressed trb-3 protects cells from starvation-mediated apoptosis in HeLa cells. In a separate study, a Q48R polymorphism in the human trb-3 was shown to impact on Akt activation in HepG2 cells [35]. This polymorphism was also associated with insulin resistance and cardiovascular risk, providing the first published human genetic clue for the relevance of trb-3 in a chronic inflammatory disease [35].

Evolution of tribbles genes

Collection of tribbles-like sequences

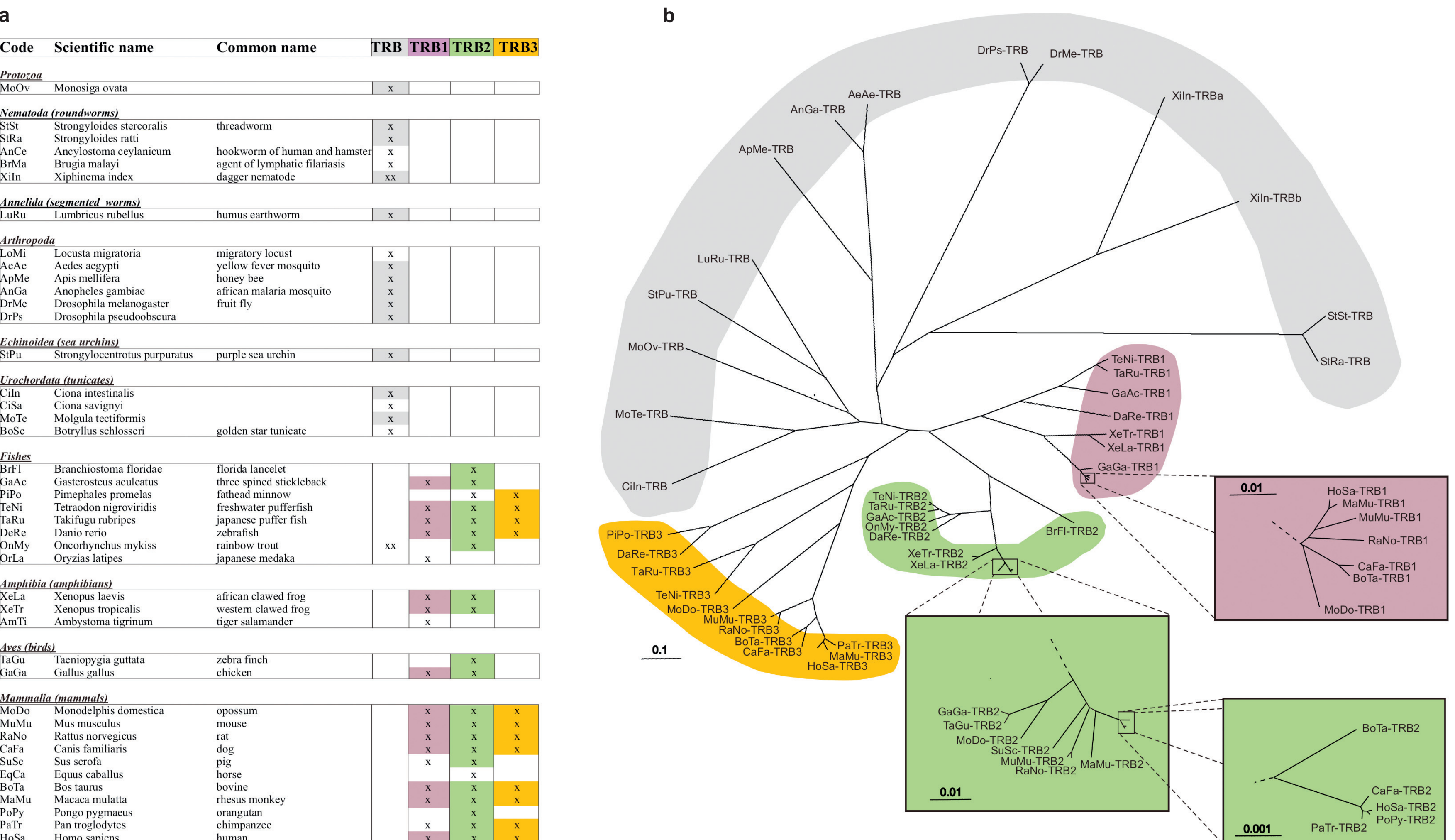
We recently undertook a systematic data mining to annotate tribbles orthologues and to characterise evolutionary relationships in this protein family between the various eukaryotic species. At the beginning of our work, approximately 40 proteins from different species were annotated as the member of tribbles family in protein sequence databases. These sequences were derived mostly from higher vertebrates but there were sequences from *amphibians* and from several fish species as well. The presence of tribbles proteins in a tunicate, *Cionia intestinalis* and in several *arthropodas*, such as different *Drosophila* species, honey bee and African malaria mosquito suggested that at least one subtype of tribbles can be traced back to earlier stages of evolution.

To refine this hypothesis and to identify the 'pop-up' point of tribbles in the evolutional history of living organisms, we performed extensive data mining in EST and genomic databases. This approach resulted in the identification of about 30 novel tribbles-related sequences (Fig. 1a), mainly from lower evolutionary categories. However, new sequences from higher organisms were also identified, where some of the tribbles sub-types were previously missing. The analysis confirmed the presence of tribbles-like sequences in roundworms, segmented worms and sea urchins. The unicellular protozoa *Monosiga ovata* represented the earliest point on the evolutional tree where a tribbles-related sequence was identified. This finding is in line with our hypothesis that tribbles are ancient proteins and may have a key role in fundamental cellular processes. However, we could not find any tribbles-like sequences in fungi, plants or prokaryotes. The overall protein homology between the newly identified tribbles sequences was sometimes very close to 35%. Below this level, the proportion of the false-positive non-tribbles-like, kinase domain-containing proteins increased very rapidly in the database searches. In such situations a new sequence was only considered to be member of the protein family if most of its closest homologues were annotated as tribbles before.

Using the sequence data collected above, we have analysed when the different tribbles subfamilies appeared during evolution. While we could only detect a single tribbles gene in invertebrates, the trb-1, trb-2 and trb-3 subfamilies began to segregate in fishes. Although the main direction of tribbles evolution seems to be clear, some findings deserve a short discussion. According to the EST data, dagger nematode (*Xiphinema index*) has two different tribbles genes. Among the fishes, four species, zebrafish (*Danio Rerio*), freshwater puffer fish (*Tetraodon nigroviridis*), Japanese puffer fish *(Takifugu rubripes)* and rainbow trout (*Oncorhynchus mykiss*) have three tribbles sequences. One sequence of *Oncorhynchus mykiss* is trb-2, but the other two could not be clearly categorized into any of the subfamilies. One of the genes of *Danio rerio*, *Tetraodon nigroviridis, Takifugu rubripes* and *Pimephales promelas* can be annotated as trb-3, which is rather unexpected, especially as neither amphibians nor birds appear to have trb-3-related genes, while mammals possess genes from all the three tribbles subfamilies. These findings might be explained by independent gene duplication events occurring in mammals and fishes followed by convergent evolution, resulting in the appearance of similar trb-3 like genes in both cases. Alternatively, mammalian trb-3 genes might have evolved directly from the fish counterparts. However, by accepting this scenario, one has to assume that trb-3 genes were lost in amphibians and birds during the evolution relatively recently. Regardless of the exact mechanisms for the emergence of trb-3, genome duplication events reported recently in many fish species [36–38] provide a plausible platform for the evolution of novel gene variants. Since many genome and EST sequencing projects are yet to be completed, it must be noted that the current description of the trb gene family (Fig. 1a) is based on a 'snapshot' of the various databases (as of September–November 2005) and it is possible that further members of the tribbles subfamilies could be revealed in some species as these sequencing programmes advance.

Evolutionary relationships in the tribbles family

Although the EST database mining gave good indications of which tribbles subfamilies are expressed at different levels of the evolutionary tree, due to the special technical attributes of the EST sequencing process, it mostly produces partial sequence fragments. We have collected all the tribbles-related EST fragments from the different species, clustered them and then assembled continuous full-ORF-containing contigs, wherever possible using TIGR Gene Indices clustering tools [39]. In most cases we achieved this target; however, a substantial fraction of our tribbles sequence collection still contained only fragments, where one or both ends of the protein-coding segment were missing. As partial sequences lead to erroneous results in phylogenetic analysis, these sequences had to be eliminated at the beginning of the process. We chose the kinase domain as a basis for the phylogenetic studies. The radial tree representation of the results is shown in Figure 1b. trb-1, trb-2 and trb-3 sequences derived from vertebrate species form three well-defined subgroups on the tree, shown in purple, green and yellow, respectively. The evolutionary distances within these subgroups are much shorter than the distances between the remaining tribbles sequences from lower evolutionary categories. By phylogenetic analysis of the kinase domain, we were unable to assign this set of ancient sequences to the vertebrate trb subgroups; therefore, we have defined a sepa-



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Code	Scientific name	Common name	TRB	TRB1	TRB2	TRB3
Protozoa						
MoOv	Monosiga ovata		x			
Nematoda (roundworms)						
StSt	Strongyloides stercoralis	threadworm	x			
StRa	Strongyloides ratti		x			
AnCe	Ancylostoma ceylanicum	hookworm of human and hamster	x			
BrMa	Brugia malayi	agent of lymphatic filariasis	x			
XiIn	Xiphinema index	dagger nematode	xx			
Annelida (segmented worms)						
LuRu	Lumbricus rubellus	humus earthworm	x			
Arthropoda						
LoMi	Locusta migratoria	migratory locust	x			
AeAe	Aedes aegypti	yellow fever mosquito	x			
ApMe	Apis mellifera	honey bee	x			
AnGa	Anopheles gambiae	african malaria mosquito	x			
DrMe	Drosophila melanogaster	fruit fly	x			
DrPs	Drosophila pseudoobscura		x			
Echinoidea (sea urchins)						
StPu	Strongylocentrotus purpuratus	purple sea urchin	x			
Urochordata (tunicates)						
CiIn	Ciona intestinalis		x			
CiSa	Ciona savignyi		x			
MoTe	Molgula tectiformis		x			
BoSc	Botryllus schlosseri	golden star tunicate	x			
Fishes						
BrFl	Branchiostoma floridae	florida lancelet			x	
GaAc	Gasterosteus aculeatus	three spined stickleback		x	x	
PiPo	Pimephales promelas	fathead minnow			x	x
TeNi	Tetraodon nigroviridis	freshwater pufferfish		x	x	x
TaRu	Takifugu rubripes	japanese puffer fish		x	x	x
DeRe	Danio rerio	zebrafish		x	x	x
OnMy	Oncorhynchus mykiss	rainbow trout	xx		x	
OrLa	Oryzias latipes	japanese medaka		x		
Amphibia (amphibians)						
XeLa	Xenopus laevis	african clawed frog		x	x	
XeTr	Xenopus tropicalis	western clawed frog		x	x	
AmTi	Ambystoma tigrinum	tiger salamander		x		
Aves (birds)						
TaGu	Taeniopygia guttata	zebra finch			x	
GaGa	Gallus gallus	chicken		x	x	
Mammalia (mammals)						
MoDo	Monodelphis domestica	opossum		x	x	x
MuMu	Mus musculus	mouse		x	x	x
RaNo	Rattus norvegicus	rat		x	x	x
CaFa	Canis familiaris	dog		x	x	x
SuSc	Sus scrofa	pig		x	x	
EqCa	Equus caballus	horse			x	
BoTa	Bos taurus	bovine		x	x	x
MaMu	Macaca mulatta	rhesus monkey		x	x	x
PoPy	Pongo pygmaeus	orangutan			x	
PaTr	Pan troglodytes	chimpanzee		x	x	x
HoSa	Homo sapiens	human		x	x	x

Figure 1. (*a*) Tribbles protein sequences ($n = 73$) from a very diverse set of organisms were collected directly from protein databases, or by the translation of EST and genomic sequences. (*b*) Phylogenetic analyses were performed using the kinase domain sequences of 60 different tribbles-related proteins. The proteins that were included in the phylogenetic studies are marked with four different colours in the sequence summary table according to their tribbles subtype. The analyses were made using Pomlk program of the Phylip package [43]. Radial tree representation were made using the graphical output of the TreeView program [44].

rate subfamily, called trb (shown in grey). In the case of *urochordata* species *Ciona intestinalis* and *Moluga tectiformis*, the phylogenic investigation of the kinase domain showed that these proteins are the member of the ancient trb subgroup, while the NT region preceding the kinase domain strongly resembles the appropriate segment of the trb-2 subgroup of the higher vertebrates (data not shown). This observation suggests that the first steps of the segregation of the modern tribbles subgroups might have happened before the appearance of vertebrates. Overall, the results suggest that a single tribbles gene was present at the early stages of the evolution and two gene-duplication events gave rise to the three modern trb subfamilies, found in fishes and mammals.

Evolutionary conserved regions in tribbles proteins

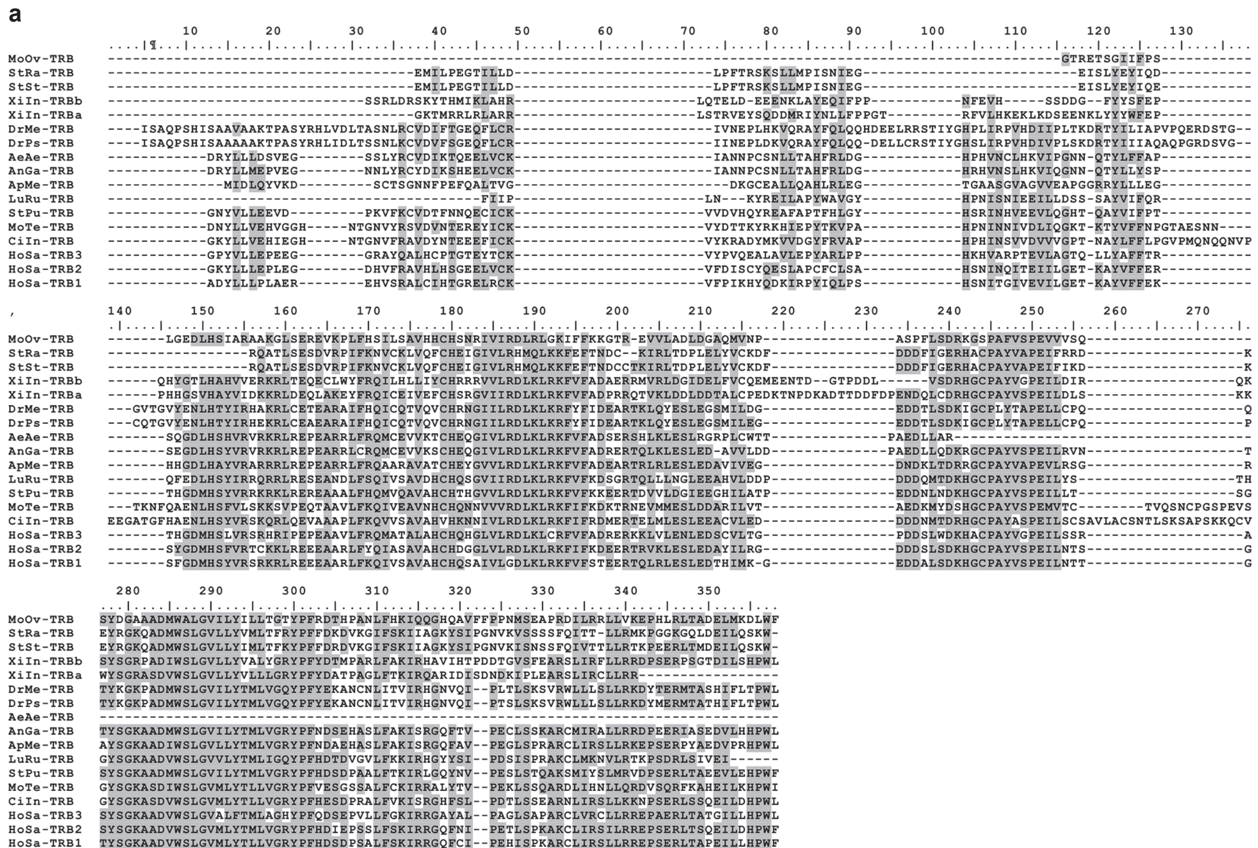
From an evolutionary prospective, members of a given protein family generally contain peptide regions that are conserved to a different degree. The identification of the most conserved domains of a complex protein family like tribbles can help to reveal some important functional constrains during the evolution. This, in turn, might help to understand the functional consequences of the different evolutionary events. A multiple alignment of the kinase-like trb domain (Fig. 2a) shows a substantial degree of sequence homology between a selected set of vertebrate and invertebrate tribbles. A similar alignment, containing all tribbles-like sequences (for the full alignment, see http://e-kisstoth.staff.shef.ac.uk/alignment.html) was used to investigate the localisation of conserved residues in many tribbles proteins and attempted to explain the potential relevance of the detected sequence patterns.

In tribbles, a kinase-like domain is located at the middle of the protein with relatively short N- and C-terminal flanking sequences. These N- and C-terminal segments proved to be the less conserved regions within the gene family. Except for very few amino acids, both fragments were very different in the tribbles subfamilies. However, some bias in the amino acid composition of the NT region with a very high serine and proline content can be considered as a general feature of the entire family (data not shown). Contrary to the diversity in the flanking regions, the kinase-like domain shows significant evolutionary conservation; however, this phenomenon manifests itself in an asymmetric manner as it is more profound in the C-terminal part of the domain. This observation is visualized in two different ways in Figure 2b, in rows A and C. In row A, the continuous black line shows the entropy (inversely proportional to the extent of conservation) of a multiple alignment containing the protein sequences of 37 kinase-like domain derived from different species and different tribbles subfamilies. In Fig. 2b, row C, we have highlighted the amino acid positions of the same multiple alignment that were invariant at least in the 90% of the aligned sequences.

Next, we localized the exon boundaries within those proteins of our sequence collection where the entire kinase-like domain was present and the genomic sequence was also known (43 sequences). With only very few exceptions, the coding sequence of the tribbles genes comprised three exons in vertebrates regardless of the subfamily classification of the coded protein. This observation strongly supports the common evolutionary origin of the trb-1, trb-2 and trb-3 subfamilies. However, in some organisms from the lower levels of the evolutionary tree, different exon structures can also be found. In the tunicate, *Cionia intestinalis,* a single tribbles protein is coded by seven exons, while the number of exons in the coding segment of tribbles gene in *Drosophila melanogaster* is not more than two (data not shown). In the vertebrate exon structure, all the three coding exons encode for parts of the kinase-like domain. Comparing the conserved regions of the kinase-like domain (described above) to the vertebrate exon structure revealed no correlation between the exon boundaries and the localisation of the non-conserved and conserved domain parts. The transition area between these is mapped to the middle of the second coding exon.

In protein kinases, the kinase domain is composed of two clear, structurally distinguishable subdomains. The smaller, N-terminal lobe contains mainly β-strands, while the larger C-terminal lobe primarily consists of α-helical structures. During the catalytic process, the enzyme reaction occurs in the space between the two lobes. The N-terminal lobe is considered to be responsible for the ATP binding, while the major function of the C-terminal lobe is the binding site of the peptide substrate [40, 41]. Since there is apparent sequence homology between the kinase-like domain of tribbles family members and other serine/threonine kinases, it seems reasonable to assume that the tribbles kinase-like domain adopts a similar conformation to the catalytic domain of the kinases. To identify the two lobes in the kinase-like domain of human trb-2 protein, we generated a homology model using the SWISS-MODEL server [42] (data not shown). By this approach we could map the N-terminal and C-terminal lobes on the protein sequence of human trb-2 (Fig. 2b) that helped to identify the positions of the two lobes in the other proteins of the multiple alignment. As demonstrated in Figure 2b, the non-conserved region of the tribbles kinase-like domain exactly covers the sequence of the N-terminal lobe, while the conserved part corresponds to the C-terminal lobe.

These observations indicate that the conserved features of tribbles proteins may be fixed more at the level of the 3-D structure rather than by the exon assembly pattern. The conservation of the C-terminal lobe does not appear exclusively within the tribbles family but is also apparent when trb proteins are compared with protein kinases.



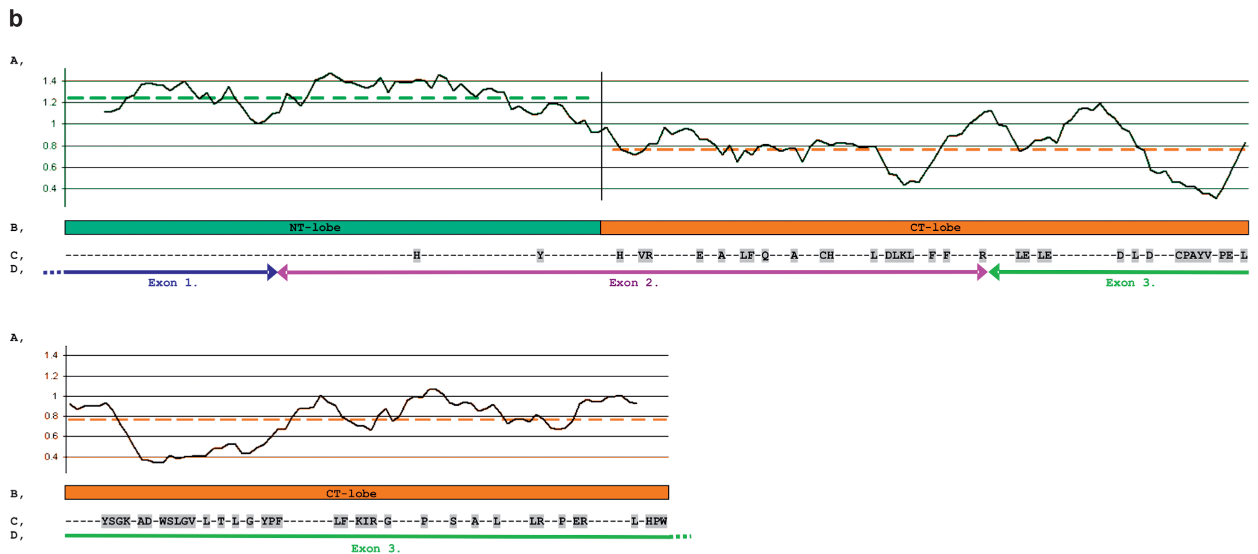


Figure 2. (*a*) Multiple alignment of the kinase domains of human trb-1, trb-2 and trb-3 polypeptides and the kinase domains of the ancient trb proteins from invertebrates. The proteins were named according the table in Figure 1a. Note that certain protein sequences in the multiple alignment are truncated since in some cases only partial sequences could be fished out from the EST databases. Identical or similar amino acids in the different columns are indicated by a coloured background. (*b*) Distribution of evolutionary conserved amino acid residues in the kinase-like domain of tribbles proteins. Row A, black line: Entropy of a multiple alignment containing of the kinase-like domain of 37 tribbles proteins derived from various organisms and different tribbles subfamilies [45]. Green dashed line: The average entropy of the multiple alignment in the region representing the N-terminal (NT) lobe of the kinase-like domains. Orange dashed line: The average entropy of the multiple alignment in the region representing the C-terminal (CT) lobe of the kinase-like domains. Row B, schematic representation of the positions of the N-terminal and C-terminal lobes in the protein sequence encoding the kinase-like domain. Row C, positions of the amino acids invariant in at least the 90% of the aligned tribbles proteins. Row D, schematic representation of the positions of the different exons participating in the coding of the kinase-like domain.

Whereas the overall protein sequence similarity is about 35% or below between the tribbles family members and the protein kinases, the local similarity of the C-terminal lobe of kinase-like domain is much higher than in the case of the N-terminal lobe or the N-terminal and C-terminal flanking regions. This is compatible with the various reports in which tribbles is characterised as a negative regulator of protein kinase-mediated signalling pathways (see above) and suggest that trb may interfere with these kinases by competing for their substrates.

In summary, both the emerging literature and the evolutionary analysis of tribbles demonstrates that these proteins are ancient members of signalling pathways regulating fundamental cellular processes. It is remarkable that they seem to be able to interact with several key intracellular signalling pathways and therefore are ideally placed as critical check points in co-ordinating cellular responses. At the same time, these proteins may also be key to the development of cell-type-specific responses, mediated by common signalling pathways. The emergence of three tribbles genes in vertebrates may reflect the increasing complexity of these organisms. As more tissue types evolve, signalling pathways have to develop ways of fine-tuning their action with a limited set of genes available. Duplication of genes with key regulatory function may be a mechanism to achieve this goal.

However, it is clear that a detailed molecular model of tribbles action is currently lacking. Although the reported interacting partners are ranging from transcription factors with both pro- (ATF4) and anti-apoptotic (p65 RelA) properties to members of the MAPK and PI3K signalling pathways, it is unclear whether these interactions take place in a cell-type- and/or stimulus-specific manner. Also, relatively few studies have investigated the function of endogenous trb. Rather, they are largely based on overexpression, which may also lead to conflicting conclusions between the different experimental systems. Clearly, the expression levels of trb relative to its interacting partners seems to be important [16, 32, 33], suggesting that further studies, possibly using recombinant proteins and *in vitro* assay systems will be needed to understand the molecular basis of tribbles action.

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