1 **Tribbles in the 21st Century: The evolving roles of Tribbles pseudokinases in**
2 **biology and disease**

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19 **Abstract**

20 The Tribbles pseudokinases control multiple aspects of eukaryotic cell biology
21 and evolved unique features distinguishing them from all other protein kinases. The
22 atypical pseudokinase domain retains a regulated binding platform for substrates, which
23 are ubiquitinated by context-specific E3 ligases. This plastic configuration has also been
24 exploited as a scaffold to support modulation of canonical MAPK and AKT modules. In
25 this review, we discuss evolution of TRIBs and their roles in vertebrate cell biology.
26 TRIB2 is the most ancestral member of the family, whereas the explosive emergence of
27 TRIB3 homologs in mammals supports additional biological roles, many of which are
28 currently being dissected. Given their pleiotropic role in diseases, the unusual TRIB
29 pseudokinase conformation provides a highly attractive opportunity for drug design.

30

31 **Keywords:** Tribbles, Trb, TRIB, TRIB1, TRIB2, TRIB3, pseudokinase, signaling, cancer,
32 evolution, ubiquitin, E3 ligase

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Introduction and Historical perspective:

Protein kinases and phosphorylation modulate all aspects of eukaryotic cell biology, and together with members of the Ubiquitin system, have become highly significant for mechanistic drug targeting [1]. Post-translational modification of proteins permits regulatory flexibility, and endows them with the ability to control signaling networks through combinatorial mechanisms. Consistently, crosstalk between phosphorylation and ubiquitination is thought to be widespread in eukaryotic signaling [2]. The three Tribbles (TRIB) pseudokinases (TRIB1, TRIB2 and TRIB3) represent a prominent sub-branch of eukaryotic pseudoenzymes that are unique within the human kinome [3, 4]. TRIB proteins regulate intracellular cell signaling, and appear to have evolved two major mechanisms of action. The first exploits conserved adaptions in the ancient eukaryotic protein kinase (ePK) fold to position protein 'substrates' and control their E3 ligase-dependent ubiquitination, likely through a switch-like pseudokinase mechanism. The second involves a more obscure scaffolding function, which operates to integrate and modulate signals flowing into and through canonical MAPK and AKT modules (Figure 1). Accordingly, TRIBs are fundamental regulators of cell cycle, differentiation, metabolism, proliferation and cell stress as reflected by the appearance of several excellent reviews in these specific areas [5-9].

Tribbles pseudokinases represent a sub-branch of the CAMK sub-family in the human kinome [3], and although a shared eukaryotic evolutionary origin is apparent from bioinformatic comparisons [10-12], the molecular basis for their specific evolutionary trajectory and associated cellular functions have not been evaluated in depth. Indeed, several critical questions in the TRIB field remain unanswered (see Outstanding Questions Box). For example, it is important to uncover clues as to why three (distinct)

TRIB pseudokinase polypeptides evolved in human cells, and how they mechanistically support diverse regulatory and disease-associated signaling pathways (Figure 1). An analysis of these issues represents a central focus of this review.

Origin and evolution of Tribbles pseudokinases

In mammalian cells, the three related Tribbles family members (TRIB1-3) are classed as serine/threonine pseudokinases that either lack (TRIB1), or possess low, (TRIB2 and TRIB3) vestigial ATP affinity and phosphotransferase capacity when analysed *in vitro* [10, 13, 14]. In many cells, TRIB 1 and 2 pseudokinases are rather unstable cellular proteins, likely due to conserved destabilizing motifs present in the small N-terminal PEST region [15, 16]. The adjacent TRIB pseudokinase domain is linked to a short C-terminal ubiquitin E3 ligase targeting motif, which has recently been proposed to interact *in cis* with the TRIB regulatory pseudokinase domain [14, 17]. In addition, the pseudokinase domain has evolved structurally [18] to correctly position and regulate potential substrates for ubiquitylation by a variety of classes of Ubiquitin E3 ligases [19]. The formation of regulated multi-protein complexes that then dictate cellular signaling is a recurring theme in Tribbles biology (Figure 1). However, while the roles of TRIB pseudokinases in cellular signaling, metabolism and disease are increasingly appreciated, little is known about their early origins and the specific signaling requirements that have shaped them during evolutionary history.

TRIB proteins derive their name from the single metazoan fly gene 'Tribbles' (Trbl) which encodes a pseudokinase with developmental roles in this model genetic organism. Trbl was discovered through three independent *Drosophila* screens, which

82 revealed genes and mutations that affect either oogenesis *via* Slbo, the alpha ortholog
83 of the mammalian CCAAT enhancer binding protein (C/EBPα) transcription factor [20] or
84 gastrulation during embryogenesis *via* String, the fly ortholog of the CDC25
85 phosphatases [21, 22]. Of major interest, Trbl mutant cells exhibit premature mitosis,
86 leading to defective gastrulation based on an inability to degrade target proteins in a
87 timely fashion. Trbl fly models continue to be instrumental in revealing requirements for
88 the pseudokinase domain in biological signaling events that have been conserved in
89 vertebrate eukaryotes [23, 24]. For example, String is the *Drosophila* ortholog of the
90 CDC25 dual-specificity phosphatases, which initiate eukaryotic S-phase and mitosis and
91 are themselves regulated at various cell cycle checkpoints. Recent data confirm that
92 TRIB2 protein expression is also cell cycle regulated in human cells, positioning it as a
93 potential modulator of CDC25 phosphatases, which it degrades through a ubiquitin and
94 proteasome-dependent mechanism [25]. Fly Trbl was also reported as an interacting
95 partner for the proto-oncogene AKT, and this interaction also appears to be conserved
96 amongst the metazoa, where TRIB3 binding directly modulates mTORC2-dependent
97 AKT activation [26-29], seemingly without affecting AKT stability. TRIB2 has been also
98 been described as a dosage-dependent suppressor of the AKT-phosphosubstrate
99 FOXO, specifically as a modulator of the cytoplasmic localization of FOXO3a in human
100 cells [30]. Interestingly, a TRIB3-regulated AKT-FOXO transcriptional network also
101 operates in human neurons [27], where TRIB3 expression levels are inversely correlated
102 with the Parkinson's disease-associated ubiquitin E3 ligase PARKIN [31]. Indeed, TRIB3
103 (but not TRIB1 or TRIB2) mRNA expression is up-regulated in the developing murine
104 brain [32] and TRIB3 immunostaining is markedly increased in sections of substantia
105 nigra from Parkinson's disease brains [31]. The relatively mild cognitive and memory

106 brain phenotypes observed in a C57BL/6 TRIB3-deficient mouse model [31] support the
107 notion that normalizing pathological increases in TRIB3 levels in neurodegenerative
108 disease might be an interesting new medical strategy.

109 To address major knowledge gaps in how the functional specialization of Tribbles
110 genes occurred during evolution, we identified and classified TRIB-related sequences
111 from all the major taxonomic groups where it is present (Figure 2). Taxonomic analysis
112 of these sequences demonstrates that they are almost entirely confined to the animal
113 kingdom, and are absent in non-metazoan eukaryote kinomes including those annotated
114 in fungi, plants and choanoflagellates. We speculate that this represents a shift towards
115 ubiquitination-based substrate regulation by the TRIB pseudokinase domains in
116 metazoan eukaryotes, although other explanations are possible.

117 As shown in Figure 2, the statistically-derived TRIB2 signature sequence is clearly the
118 earliest (most ancestral) member amongst the TRIB1, 2 and 3 family. Consistently, clear
119 orthologs of TRIB2 (but not TRIB1 or TRIB3) can readily be detected in the oldest
120 metazoans such as cnidarians (Nematostella vectenisis) and sponges (Amphimedon
121 queenslandica), whereas TRIB1 and TRIB3 orthologs are restricted to specific later
122 metazoan lineages that led to the vertebrates, where all three TRIBs pseudokinases are
123 consistently preserved. It is likely that the emergence of TRIB1 and TRIB3 at later
124 stages of evolution was driven by unique regulatory requirements associated with
125 signaling integration by these proteins in higher organisms. TRIB1 likely appeared
126 through TRIB2 gene duplication during the diversification of vertebrates from
127 invertebrate, since multiple copies of TRIB2 and a single copy of TRIB1 are observed in
128 non-mammalian chordates such as fish (*actinopterygii*) and reptiles. Remarkably, the
129 TRIB3-specific sequence signature appears in all mammalian lineages, and from the

130 extensive data analysed here, emerged relatively recently during the diversification of
131 mammals from non-mammalian chordates (Figure 2). While most mammalian species
132 harbor at least one copy of TRIB1, 2 and 3, some encode multiple copies, suggesting
133 functionally-relevant gene duplications; an extreme version being the marmoset
134 *Callithris jacchus*, whose genome encode three copies of TRIB1, one copy of TRIB2,
135 and 2 copies of TRIB3. The cellular consequences of these duplications are obscure,
136 although we speculate that they represent a requirement for new biological functions
137 associated with higher vertebrates (perhaps including unique TRIB3 roles in highly-
138 evolved brains), which might be driven by increased gene dosages of this specific
139 pseudokinase. Whatever their specific functions, Tribbles genes have likely been co-
140 opted into evolutionary-appropriate biological roles that depend on their unique
141 pseudokinase properties when compared to catalytically active kinases (Box 1), which
142 we and others have defined previously [3, 10, 13, 33-36].

143 **What defines a Tribbles pseudokinase?**

144 TRIB pseudokinases are predicted to be three-domain proteins containing an N-
145 terminal PEST region, a pseudokinase domain (containing an unusual N-lobe and a
146 canonical C-lobe) and a C-terminal COP1 binding peptide region, which interacts *in cis*
147 with a pocket formed adjacent to the unusual αC-helix found in the TRIB pseudokinase
148 domain (Figure 1). Recent structural analysis of the human TRIB1 pseudokinase domain
149 [14] confirms an atypical kinase fold that diverges most notably in the N-lobe, which
150 harbours most of the catalytic machinery, but which preserves a putative substrate-
151 binding site in the C-lobe of the kinase domain. No structural information has been
152 reported for the N-terminal PEST domain, or for any domains of TRIB2 or TRIB3

153 pseudokinases. Although these same defining regions are conserved across TRIB
154 polypeptides when comparing the pseudokinase domain with catalytically active CAMK
155 relatives (Box 1), the availability of tens of thousands of protein kinase like sequences
156 from diverse organisms provides a timely opportunity to define distinguishing or unique
157 features of TRIB pseudokinases using statistical comparisons of very large datasets.
158 Such approaches across kinomes have previously been invaluable to provide new
159 insights into protein kinase structure, function and evolution [37-42]. As collated in
160 Figure 3, a Bayesian statistical comparison reveals strong selective constraints imposed
161 on *each of the individual* TRIB kinase sequences during evolution. At a gross level,
162 these constraints correspond to 'TRIB-specific' residues/motifs that are highly conserved
163 in TRIB kinases but strikingly different in the quite closely-related pseudokinase SgK495
164 and the broader family of canonical CAMKs. We focus on the most distinctive features in
165 the core (conserved) regions of the TRIB family, namely the pseudokinase domain
166 (Figure 3A) and flanking C-terminal tail (Figure 4A). In passing, we note that the N-
167 terminal PEST domain contains distinct sequence motifs are characteristic of each TRIB
168 variant, and these sub-family specific motifs are assumed to regulate distinct protein
169 turnover patterns in both 'normal' and disease-associated cells [43, 44].

170 Within the pseduokinase domain, TRIB-specific constraints are imposed on
171 residues/motifs that are dispersed in primary sequence, but that spatially cluster in two
172 critical regions of the pseudokinase domain (Figure 3A), namely the active site and C-
173 terminal substrate-binding lobe. The divergent nature of active site residues in TRIB
174 pseudokinases have been noted in previous studies and in the recently solved crystal
175 structure of TRIB1 [14], where these residues mediate specific hydrogen bonding and
176 van der Waals interactions (Figure 3B) that stabilize the activation loop in a unique

177 inactive conformation that serves to preclude nucleotide binding in the TRIB1 ATP
178 binding site [14]. In contrast to the pseudo-active site, TRIB-specific divergence in the C-
179 lobe has not been evaluated in depth, despite the overall structural similarity of TRIB1 to
180 the C-lobe of canonical CAMKs. Some of the TRIB-specific residues of unknown
181 functions in the C-lobe include the D[KR]H[GA]C motif in the activation loop, H138 in the
182 D-helix and a conserved cysteine residue in the αH helix (Figure 3B, C). In the crystal
183 structure of TRIB1, the region corresponding to the D[KR]H[GA]C motif in the activation
184 segment is disordered. However, modeling of the activation loop in both an active and
185 inactive conformation suggests that TRIB-specific residues in the C-lobe potentially
186 stabilize the activation loop in an auto-inhibitory conformation that might occlude
187 substrate binding, as previously revealed by structural analysis of TRIB1 [14]. The C-
188 lobe is a universal docking site for protein substrates in canonical kinases and auto-
189 inhibitory conformations involving the activation loop have been observed in multiple
190 kinases, including canonical CAMKs [45]. Thus it is likely that a near-universal regulatory
191 kinase mechanism is also at play in the TRIB pseudokinases, and that TRIB-specific
192 constraints in the pseudokinase domain reflect specific variations on a very common
193 mechanistic auto-regulatory theme common found in most, if not all, kinases.

194 **A unique C-terminal regulatory region in all TRIB pseudokinases.**

195 In addition to characteristic residues/motifs in the pseudokinase domain, the critical C-
196 terminal tail involved in protein:protein interactions is readily identified as a
197 distinguishing feature of TRIB kinases; this segment is highly conserved in TRIB kinases
198 but strikingly different in kinases outside of the TRIB subfamily (Figure 4A). In fact, no
199 detectable similarity is observed at this locus between TRIB primary sequences and any

200 other evolutionarily-related protein kinases, clearly defining the tail as a statistically-
201 unique feature. The TRIB C-terminal tail is defined by two unique sequences: the
202 HPW[F/L] and DQXVP[D/E] motifs near the extreme N and C-terminal regions of the C-
203 tail, respectively (Figure 4A). The HPW[F/L] motif is involved in binding of MEK1 (and
204 other MAPKK dual specificity kinases) to TRIB pseudokinases [46-48] whereas the
205 DQXVP[D/E] motif is intimately involved in COP1 binding in all TRIB proteins [49, 50],
206 and absolutely required to drive tumourigenesis in leukemia models [51]. Although these
207 motifs are not observed in the C-terminal tail of other protein kinases, they are predicted
208 to engage with the TRIB pseudokinase domains in a manner analogous to canonical,
209 well-studied protein kinases such as PKA and MAP kinases, which utilize these flanking
210 regions to drive regulatory mechanisms involved in kinase activation and substrate
211 phosphorylation (Figure 4B). This common regulatory theme, evident between a
212 divergent pseudokinase family (modeled as TRIB2) and multiple canonical kinase
213 families in which allosteric coupling is established (represented by AGC kinases and
214 MAP kinases), re-enforces how flanking sequences outside the (pseudo)kinase domain
215 appear to have been repeatedly employed to permit switch-like regulation of the kinase
216 fold. Of particular interest for the TRIBs, this mechanism is predicted to be independent
217 of catalysis, although the phosphorylation of a highly specific localized cellular substrate
218 is rather challenging to rule out completely.

219 Interestingly, the HPW[FL] motif is tethered to the C-lobe of the pseudokinase
220 domain in the recently solved TRIB1 crystal structure, whilst the DQXVP[D/E] motif is
221 tethered to the N-lobe. Why would such tethering of the C-tail to the pseudokinase
222 domain be important for TRIB functions? One possibility is that this interaction allows
223 efficient coupling of COP1 binding (or other regulatory binding proteins) in the C-tail with

224 co-opted substrate-binding functions associated with the degenerate pseudokinase
225 domain. Such a view may also explain why the C-helix and activation loop conformation
226 in the TRIB1 (and presumably TRIB2 and TRIB3) pseduokinase domain are stabilized in
227 a unique conformation [14], since potential conformational changes in these regions can
228 be allosterically coupled to the COP1 binding site, which is absolutely required for
229 substrate ubiquitination. Indeed, the binding of a TRIB1 C-tail-derived peptide sequence
230 to a COP1-motif peptide is of a measurably higher affinity when compared side-by-side
231 with a TRIB1 protein containing the C-tail [18], the latter presumably retaining coupled
232 pseudokinase and C-tail interactions. Thus protein allostery, rather than
233 phosphorylation-based catalysis more commonly associated with canonical protein
234 kinases, might be the key driving force for TRIB kinase evolution and functional
235 specialization. In turn, this suggests that TRIBs evolved to be pseudokinases that exploit
236 a non-canonical (but still bilobal) protein kinase fold that modulates cellular ubiquitination
237 through protein:protein interactions. The ingenious re-use of a pseudokinase domain to
238 mediate signalling has also been noted in other evolutionary-distinct pseudoenzymes,
239 including the PAN3 and ADCK3 pseudokinases, which have refined the kinase fold to
240 scaffold enzyme-catalyzed processes as diverse as mRNA deadenylation [52] and
241 Coenzyme Q biosynthesis [53]. In support of a co-evolutionary hypothesis in TRIB
242 pseudokinases, it is interesting to note that some TRIB2 orthologs (such as a
243 pathogenic thread worm) lack detectable sequence similarity in the C-tail sequence, and
244 also diverge in complementary C-tail docking regions in the pseudokinase N-lobe
245 (modeled as deletions in alignment Figures 3A and 4A). Unfortunately, since parasitic
246 threadworms kinomes are currently poorly annotated, we cannot rule out that these
247 sequences either exist, or have become cryptic at the amino acid level. Nonetheless, we

248 suspect that characterizing unusual TRIB variants and potential mechanisms of action in
249 a variety of model organisms will be important for fully understanding TRIB functions in
250 both normal human biology and disease.

251 **Tribbles links to cancer: A corruption of cell signalling?**

252 In humans, the Tribbles gene family have been implicated in many different
253 cancers, but especially in melanoma, lung, liver and acute leukaemias [5, 6]. The
254 molecular basis of these disease links is still in the process of being dissected in a
255 variety of cell types and model systems. However, a major mechanistic function of
256 TRIBs in cancer cells appears to be the (inappropriate) association of TRIB proteins with
257 substrate degradation and stability networks, leading to a subsequent imbalance in
258 timely regulation of crucial transcriptional networks. For example, TRIB2-mediated
259 degradation of the transcription factor C/EBPα is known to have an oncogenic role in the
260 development of acute myeloid leukemia (AML) [54, 55], lung [56] and liver cancers [57,
261 58]. These studies all point to abnormal regulation of TRIB transcription, translation or
262 protein turnover as disease drivers, and below we use the hematological system and
263 other cancer models to describe how this is thought to work mechanistically at the
264 cellular level in mammals.

265 **TRIB pseudokinase function in the myeloid and lymphoid systems.**

266 The Trib2 gene was first identified as a murine myeloid oncogene, since its
267 overexpression in a bone marrow transplant model leads to the development of a potent
268 transplantable AML with 100% penetrance and short latency [54]. In this model, TRIB2
269 preferentially degrades the p42 isoform of the myeloid transcription factor C/EBPα,
270 which leaves the truncated oncogenic p30 isoform (which lacks the canonical TRIB-

271 binding site identified in the TRIB1 crystal structure) intact [54]. TRIB1 (but not TRIB3)
272 functionally resembles TRIB2 in this phenotypic mouse cancer model, since it also
273 degrades C/EBPα and causes highly penetrant AML (Figure 5), in-line with a postulated
274 TRIB evolutionary pathway that led linearly between TRIB2 and TRIB1 and hence on to
275 TRIB3 (Figure 2) and the high level (71%) of amino acid between TRIB1 and TRIB2
276 within the pseudokinase domain. The functional difference between TRIB
277 pseudokinases in these systems is further highlighted by their differential expression in
278 haemopoietic cells [59] with TRIB2 highest in the lymphoid cell compartment, TRIB1
279 highest in the myeloid cell compartment, and TRIB3 expression constant across all cell
280 types examined. Interestingly, the human *TRIB1* gene is located at the same
281 chromosomal locus (8q24.13) as the *MYC* oncogene, and *MYC* (and potentially *TRIB1)*
282 cancer susceptibility genes are therefore co-amplified in a significant percentage of
283 human tumours, where the 8q24 amplicon is the most commonly amplified region
284 across multiple cancer types [60]. Furthermore, an 'oncogenic' R107L TRIB1 mutation in
285 the pseudokinase domain has been identified in a Down syndrome-related AML [7]; this
286 Arg residue is broadly conserved across the TRIB pseudokinases (Figure 3A, asterisk),
287 but is a Leu residue in the canonical kinase PKA, where it packs up against hydrophobic
288 residues in the C-terminal tail [39]. Interestingly, when this amino acid is analysed across
289 the kinome [61], an Arg or Lys residue is found in ~35% of human kinases, with Leu
290 representing the most common single amino acid, accounting for ~25% of
291 (pseudo)kinases in the human ePK family. Although mechanistic effects of an R107L
292 substitution are not fully understood in TRIB1, enhancement of both ERK
293 phosphorylation and C/EBPα degradation have been demonstrated in an R107L TRIB1
294 murine bone marrow of AML [62]. Based on the crystal structures of PKA and TRIB1, we

295 speculate that this mutation contributes to abnormal TRIB1 pseudokinase function by
296 disrupting regulatory protein interactions with interacting partners, such as MAPKK
297 family members [46], Ubiqutin E3 ligases or *cis*-acting flanking regulatory segments in
298 TRIB itself**.** Of related interest, TRIB1 gene polymorphisms are also associated with
299 nonalcoholic liver and metabolic syndromes [63], consistent with a relatively specialized
300 function for TRIB1 in lipid metabolism [8, 43].

301 In the hematopoietic system C/EBPα is essential for granulopoiesis [64] and
302 TRIB1 and TRIB2-mediated degradation of C/EBPα p42 blocks this differentiation
303 process. Mechanistically, degradation of C/EBPα p42 by TRIB2 is known to occur *via* a
304 proteasome dependent pathway involving lysine 48 polyubiquitination [55]. Using mouse
305 genetics it has been shown that the presence of C/EBPα is paradoxically required for
306 TRIB2 induced AML, and only in the presence of the C/EBPα p42 isoform is a
307 cooperative effect observed with TRIB2 and C/EBPα p30 [55]. Structure-function
308 analysis of TRIB2 revealed that deletion or mutation of the TRIB signature C-terminal E3
309 ligase COP1-binding site (Figure 4A) prevented TRIB2-mediated degradation of C/EBPα
310 and this correlated directly with a failure to induce AML *in vivo* [51]. Interestingly, TRIB1,
311 2 and 3 all retain COP1-binding motifs in their C-tails (Figure 4A), but TRIB3 alone fails
312 to drive C/EBPα degradation. This finding is consistent with a unique (or only partially
313 overlapping) set of target substrates for TRIB3 in comparison to TRIB1 and TRIB2.
314 However, preserved functionality in the C-tail in TRIB3 is confirmed by its ability to
315 facilitate cellular COP1-mediated ubiquitination and degradation of Acetyl CoA
316 carboxylase, the rate-limiting enzyme in fatty acid synthesis [50]. Distinct ubiquitin E3
317 ligases have also been associated with TRIB2 signaling, including TRIM21 in a lung

318 cancer model [56]. In liver cancer models, TRIB2 overexpression conversely stabilizes
319 the co-activator YAP, an oncogenic transcription factor, *via* binding to the distinct E3
320 ubiquitin ligase β-TRCP [65, 66]. Intriguingly, and in contrast to COP1 and TRIM21,
321 TRIB2 interaction with β-TRCP leads to inhibition instead of promotion of substrate
322 degradation; it remains unclear if post-translational modifications control the targeted
323 interaction of different TRIB pseudokinases with specific ubiquitin ligases. However, in
324 terms of feedback regulation, TRIB2 stability has also been shown to be regulated at the
325 protein level in liver cancer cells, in part due to the downregulation of the E3 ligase
326 SMURF1 [16].

327 Through their interaction with ubiquitylated transcription factors, or regulation of
328 signaling pathways that culminate in cell-type specific reprogramming, TRIB
329 pseudokinases are regulated by, and fundamental regulators of, gene transcription
330 networks. An analysis of TRIB1, 2 and 3 promoters confirms that TRIB1 and 2 both
331 possess putative E2F and C/EBPα transcription factor binding sites amongst a large
332 number of conserved canonical transcription factor sequence determinants (Box 2).
333 Consistently, TRIB2 has been shown to be part of a regulatory loop involving E2F1 and
334 TRIB2, in which E2F1 binds to the *TRIB2* gene promoter to drive expression of the
335 TRIB2 protein, which is then positively reinforced via p30 C/EBPα expression of E2F1
336 [67]. In T cell leukemia models, the Paired homeobox transcription factor PITX1 was
337 shown to regulate TRIB2 [68]. In addition, aberrant TAL-1 activation, which is detected
338 in up to 60% of T-ALLs [69], was shown to transcriptionally target TRIB2 [70], allowing
339 TRIB2 to positively reinforce an oncogenic transcriptional programme involving GATA3,

RUNX1, MYB and E2A, potentially balancing the oncogenic and tumour suppressive biological activities of these factors [71].

Despite being capable of driving myeloid leukaemia when overexpressed alone, it is assumed that cooperating lesions occur in TRIB1 and TRIB2 mouse leukaemia models (Figure 5). Putative cooperating genes in TRIB1 and TRIB2-mediated AML include HOX pathway genes such as HOXA9, MEIS1, NUP98-HOXD13 and C/EBPα p30 [48, 55, 72, 73]. Gene expression analysis has revealed that *TRIB2* expression levels, while generally low in AML, are higher in *PML-RARα* positive leukaemia than *PML-RARα* negative leukaemia [59]. As described, the TRIB1 gene is located on chromosome 8, the most common chromosomal gain in human AML and APL (distinguished by the fusion oncogene PML/RARA). The hypothesis that TRIB1 and PML/RARA cooperate has been tested, and while they do not cooperate to drive a shorter latency leukaemia *in vivo*, TRIB1 and PML/RARA have functionally redundant inhibitory effects on C/EBPα, which also impacts responses [74] to therapeutic All Trans Retinoic Acid (ATRA, Figure 5).

The association of TRIB2 with a subset of AMLs with dysregulated C/EBPα is especially intriguing, since T-lymphoid genes including *NOTCH1* [75] and *CD7*, as well as *CD34*, a stem cell gene, also associate with this subset [76]. It is interesting to speculate that TRIBs may have a role in lineage decisions or phenotypic characteristics of myeloid and lymphoid leukaemias, which may be determined by their specific cellular substrates. While it was shown that TRIB2 is a transcriptional target of the T cell transcription factor NOTCH1 [76], the absence of TRIB2 in a murine TRIB2 knockout model [77] was found to actually accelerate NOTCH1-driven T-ALL (Figure 5). This work

363 also revealed a novel tumor suppressor-like function for TRIB2 in cell cycle and
364 proliferation of early T cell progenitor cells, and associated high levels of TRIB2
365 expression with early immature T-ALL and deregulated MAPK signalling [77]. TRIB2 is
366 cyclically expressed during the cell cycle and has the ability to promote the proteasomal
367 degradation of the mitotic regulator CDC25C, which might explain some of the
368 uncontrolled proliferation characteristic of leukaemia [25]. It was subsequently confirmed
369 in an independent study that this tumour suppressor activity of TRIB2 is lymphoid-cell
370 specific [78].
371

372 **TRIB pseudokinase functions in other model cancer systems.**

373 Further evidence for TRIB2 as a modulator of tumorigenic activity comes from
374 liver cancer cells, where the overexpression of TRIB2 was shown to negatively regulate
375 WNT signallilng activity, leading to inhibition of cell growth [19]. The cell models in this
376 study possess wild type (WT) β-catenin, and the authors confirmed that TRIB2
377 overexpression reduced WNT-mediated transcriptional activity and decreased levels of
378 β-catenin and TCF4 protein. Consistent with this model, the loss of TRIB2-impaired liver
379 cancer cell survival *in vitro* and *in vivo* [66] is associated with a dominant β-catenin
380 mutation. Interestingly, the broad analysis of all cancers failed to identify conserved
381 mutations or chromosomal alterations involving TRIB2. In contrast, its elevated
382 expression is strongly associated with cancer prognostics [79]. Indeed, *TRIB2* has been
383 identified as a candidate biomarker for melanoma diagnosis and progression as it
384 exhibits low expression in healthy skin samples, increases in benign melanoma, with
385 highest expression seen in malignant melanoma samples [80]. In addition, TRIB1

386 expression has been shown to be essential for the survival of prostate cancer cells and
387 is linked with an aggressive disease phenotype and poor prognosis [81]. TRIB1 is also
388 involved in the etiology of glioma [82], breast [83], ovarian [84] and follicular thyroid
389 cancer [85]. TRIB3 expression was found elevated in colorectal cancer patients and its
390 expression correlated with poor overall survival [86]. *TRIB3 gene* expression, in contrast
391 to TRIB3 protein expression [87], has been shown to correlate with poor prognosis in
392 breast cancer patients [88] and a poor prognosis in NSCLC [89].
393

394 **Concluding Remarks:**

395 The appearance and retention of pseudoenzymes during evolution in essentially all
396 enzyme families suggests that these domains are malleable templates that can be co-
397 opted for new biological functions when required [90,91,92]. This is very clearly
398 demonstrated within the human protein kinase superfamily by the three TRIB
399 pseudokinases, which together control large networks of cellular signaling pathways,
400 many of which are known to be dysregulated in disease. This review highlights the
401 importance of classifying and analyzing each TRIB family member as a unique
402 pseudokinase variant, and this is most clearly shown by comparing their evolutionary
403 and disease-associated biology in vertebrates. The evolution of TRIB1 and TRIB3 from
404 a common TRIB2 ancestor is particularly interesting, and by employing unique structural
405 and mechanistic features, Tribbles pseudokinases appear to have evolved a set of
406 fundamental biological roles, some of which are shared, at least in the simplistic
407 experimental cell and animal models evaluated thus far. In the future, proteomic and cell
408 biology approaches will permit the accurate dissection of the common and specific TRIB
409 machinery that brings about the three TRIB pseudokinase signaling modules. The

410 mechanisms that underlie TRIB proteins ability to function act as oncogenes or tumour
411 suppressors are currently not well understood. However, these are likely to be linked to
412 their complex functions in cell proliferation, protein degradation, transcriptional
413 regulation and canonical signaling pathway modulation and might also be cell-context
414 dependent, impacting on the cellular fate of both normal and tumour cell fate. Indeed,
415 these signalling pathways, and the TRIB pseudokinases and protein:protein interactions
416 that regulate them, present new and potentially important pharmacological opportunities
417 for therapeutic intervention [93, 94] in both metabolic and proliferative disorders.
418

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426 **Box and Figure Legends**

427 **Box 1. CAMK sub-family sequence alignment within the (pseudo)kinase domain.**

428 (A) Alignment and Sequence Logo amino acid conservation plot of Drosophila Tribbles
429 (Trbl), human TRIB1-3, human CASK (a CAMK-related pseudokinase), canonical
430 human CAMK1α, CAMK2γ and the benchmark PKAcβ AGC kinase domains. A
431 comparison of sequences around the degraded TRIB Gly-rich loop, the conserved β3
432 Lysine, the unusual TRIB catalytic loop and the metal-binding 'DFG' motif (including the

433 TRIB-specific E[S/N]LED sequence) are boxed in red. Conserved amino acid motifs at
434 these positions together define the TRIB pseudokinase domain signature (applied in
435 Figures 2 and 3). The HPW[F/L] MEK binding site at the junction of the TRIB
436 pseudokinase C-lobe and C-tail is boxed in blue. Numbering refers to amino acid
437 boundaries within each human (pseudo)kinase domain.

438

439 **Box 2. Predicted transcription factor binding sites in human and murine *TRIB***
440 **gene promoter regions.**

441 Transcription factor binding sites were predicted using Transcription Element Search
442 System (TESS) software [95], based on the sequence of the extended 5'UTR regions
443 sequenced from human and murine TRIB1 (A), TRIB2 (B) or TRIB3 (C) genetic loci.
444 Twelve canonical transcription factor binding sites identified in the two sets of promoters
445 are colored according to the key. Note that several functional E2F1 and C/EBPα binding
446 sites on the TRIB2 promoter predicted in this analysis have been experimentally verified
447 [67]. The differences in length of the 5'UTR region of each promoter analysed are
448 indicated for reference. Note the relatively large predicted number of transcription factor
449 binding sites for TRIB2 compared to the relatively small number of predicted
450 transcription factor binding sites for TRIB3.

451 **Figure 1. Classical Tribbles signaling features.**

452 All TRIB polypeptides contain a variable N-terminal 'PEST' domain, a pseudokinase
453 domain lacking a canonical 'DFG' (metal-binding) motif, and a unique C-tail, which
454 contains two key regulatory elements. The HPW[F/L] motif at the beginning of the

455 pseudokinase C-tail targets MAPKK/MEK family members, giving Tribbles the potential
456 to regulate and/or integrate distinct stress and proliferative MAPK modules. The
457 conserved structural C-terminal DQXVP[D/E] peptide motif supports a direct association
458 with E3 ubiquitin ligases including COP1, which specifies K48-linked ubiquitin chains in
459 substrates such as C/EBPα, thereby regulating transcription factor stability *via* the
460 ubiquitin proteasome system. TRIBs can also modulate AKT/FOXO signaling modules,
461 although the molecular details for individual pseudokinases remain to be clarified.
462 Overall, a series of shared mechanisms contribute to TRIB-regulated signaling pathways
463 that support cell context-specific programmes of cellular differentiation, proliferation and
464 survival. P=phosphate, Ub=Ubiquitin

465 **Figure 2. Taxonomic coverage and analysis of TRIB family kinases**.

466 The number of TRIB1, TRIB2, and TRIB3 orthologs detected across all the major
467 metazoan species for which accurate genomic kinome data is available are shown.
468 Each row represents a single species and the number of TRIB othologs identified in
469 each species is indicated. Orthologs were identified by scanning a hierarchical
470 sequence profile of diverse ePKs and TRIB family members against sequenced
471 proteomes contained in the latest non-redundant proteome sequence set in Uniprot
472 (Downloaded October 2016) using the MAPGAPS program [96]. The most significant
473 hits to TRIB1, TRIB2 and TRIB3 profiles were annotated as putative orthologs.
474 Fragmentary sequences of less than 150 amino acids in length were filtered out.
475 Reptilian TRIB3 orthologs were detected in larger sequence databases (NCBI nr, EST)
476 and all three TRIB pseudokinases are observed in reptilian species (including alligator)
477 in the corresponding Uniprot proteome sequence set.

478 **Figure 3. Distinguishing sequence and structural features of TRIB pseudokinases**
479 **(A)** Constraints that help distinguish TRIB kinases from all other kinases are shown in a
480 contrast hierarchical alignment, where representative TRIB kinases from diverse
481 organisms constitutes the display alignment; all TRIB-like sequences available (492
482 sequences, September 2016) constitute the foreground alignment, and related CAMK
483 sequences (79,487 sequences) constitute the background alignment. Complete
484 foreground and background alignments are not shown due to the hundreds of text pages
485 required. Instead, information encoded in these large alignments is shown as residue
486 frequencies directly below the display alignment where, for example, the number 5
487 indicates that the corresponding residue occurs 50–60% of the time at the
488 corresponding position. The histogram above the alignment plots the strength of the
489 selective pressure shifting residues at each position in the TRIB kinases away from the
490 residue composition observed at the corresponding positions in CAMKs. Residue
491 positions subject to the strongest constraints are highlighted with chemically similar
492 amino acids colored similarly; very weakly conserved positions and non-conserved
493 positions are shown in dark and light gray, respectively. Dots below the histograms
494 indicate those residues positions that most strikingly distinguish TRIB kinases from
495 CAMKs as selected by the Bayesian pattern partitioning procedure [97]. Key secondary
496 structural elements are indicated above the alignment; amino acid numbering
497 corresponds to human TRIB2. Identifiers for TRIB sequences used in the display
498 alignment can be compared to canonical kinase sequences by inspecting Box 1. **(B,C)**
499 Modelling of structural disposition of TRIB-specific residues forming the regulatory
500 activation loop, atypical DFG motif (E[S/N]LED) and αC helix in human TRIB2. The
501 kinked αC-helix is shown in yellow and the activation loop (A-loop) is shown in magenta.

502 TRIB family conserved residues are shown in green. Putative hydrogen bonding
503 interactions in the modeled structure are shown by dotted lines and the putative
504 substrate-binding site in the C-lobe is labeled. Structural image was generated using
505 PyMoL. Specific TRIB Gene Identifiers are listed in the Legend to Figure 4.

506

507 **Figure 4. The C-tail, a unique 'degrading' feature of TRIB pseudokinase**
508 **polypeptides.**

509 (**A)** Alignment of TRIB pseudokinase C-tail segment highlighting key conserved residues
510 and motifs found in eukaryotic TRIB pseudokinases. See Figure 2 legends for details**.**
511 Unique TRIB gene identifiers are: Trib1 Human Q96RU8, Trib1 Mouse Q8K4K4, Trib1
512 Cow A6QLF4, Trib1 Elephant G3T9X9, Trib1 Chicken H9L0P6, Trib1 Green anole
513 G1KJZ8, Trib1 Alligator A0A151P7Z3, Trib1 Frog F7BWB1, Trib1 Coelacanth
514 H3ALB4, Trib1 Zebrafish E7FD70, Trib2 Human Q92519, Trib2 Mouse Q8K4K3, Trib2
515 Cow Q5GLH2, Trib2 Elephant G3TC04, Trib2 Chicken Q7ZZY2, Trib2 Green anole
516 G1K8G5, Trib2 Alligator A0A151N8V7, Trib2 Frog Q76D08, Trib2 Coelacanth
517 H3A37,Trib2 Zebrafish E7F3S2, Trib2 Fire ant XP_011171156.1,Trib2 Acorn Worm
518 XP_002742313.1, Trib2 Threadworm A0A0K0E067; Trib2 Sea hare
519 XP_005101496.1, Trib2 Sea urchin XP_792075.2, Trib2 Sponge I1G1T0, Trib3 Human
520 Q96RU7, Trib3 Mouse Q8K4K2, Trib3 Cow Q0VCE3, Trib3 Elephant G3SZ76,
521 Trib3 Alligator A0A151NVV1. **(B)** Common mechanism for structural tethering of the C-
522 tail to the kinase domain in TRIB2 (model based on TRIB1 X-ray analysis, PDB ID:
523 5CEM) and two canonical kinase families, with PKA representing the AGC kinases [39]
524 and ERK2 representing the MAP kinases [98]. All three sub-families of (pseudo)kinase

525 are regulated by conformational changes in the C-terminal tail that directly engage the
526 kinase domain *in ci*s.

527 **Figure 5. TRIB1 and TRIB2 pro- and anti-tumorigenic activities associated with**
528 **vertebrate leukaemias.**

529 Top panel depicts murine cancer models in which TRIB1 and TRIB2 function as
530 oncogenes when TRIB1 or TRIB2 are overexpressed (+TRIB1, +TRIB2) leading to fully
531 penetrant, fatal myeloid (AML, green), but not lymphoid (T-ALL) leukemias. Elevated
532 TRIB1 expression also contributes to chemotherapy resistance (a pro-tumorigenic
533 response) in APL (indicated in a PML/RARA+MYC model). Bottom panel depicts mouse
534 models in which the loss of TRIB2 reveals a tumour suppressive activity. This is shown
535 through homozygous TRIB2 knockout, which results in an accelerated lymphoid (T-ALL
536 induced by active NOTCH1, red), but not myeloid, leukaemia. WT=wild type mouse.
537 BM=bone marrow. AML = acute myeloid leukaemia. APL = acute promyelocytic
538 leukaemia (a subtype of AML). T-ALL = T cell acute lymphoblastic leukaemia. Cebpa$^{-/-}$ =
539 C/EBPα knockout mouse. Trib2$^{-/-}$ =Trib2 knockout mouse. ATRA = all-trans-retinoic-acid,
540 an APL therapy**.** Citations refer to *in vivo* mouse models of leukemia demonstrating
541 oncogenic or tumour suppressive TRIB biology.

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References

1. Cohen, P. and M. Tcherpakov, *Will the ubiquitin system furnish as many drug targets as protein kinases?* Cell, 2010. **143**(5): p. 686-93.
2. Hunter, T., *The age of crosstalk: phosphorylation, ubiquitination, and beyond.* Molecular cell, 2007. **28**(5): p. 730-8.
3. Manning, G., et al., *The protein kinase complement of the human genome.* Science, 2002. **298**(5600): p. 1912-34.
4. Boudeau, J., et al., *Emerging roles of pseudokinases.* Trends Cell Biol, 2006. **16**(9): p. 443-52.
5. Lohan, F. and K. Keeshan, *The functionally diverse roles of tribbles.* Biochemical Society transactions, 2013. **41**(4): p. 1096-100.
6. Yokoyama, T. and T. Nakamura, *Tribbles in disease: Signaling pathways important for cellular function and neoplastic transformation.* Cancer science, 2011. **102**(6): p. 1115-22.
7. Nakamura, T., *The role of Trib1 in myeloid leukaemogenesis and differentiation.* Biochemical Society transactions, 2015. **43**(5): p. 1104-7.
8. Iwamoto, S., et al., *The role of TRIB1 in lipid metabolism; from genetics to pathways.* Biochemical Society transactions, 2015. **43**(5): p. 1063-8.
9. Johnston, J., et al., *Tribbles in inflammation.* Biochemical Society transactions, 2015. **43**(5): p. 1069-74.
10. Bailey, F.P., et al., *The Tribbles 2 (TRB2) pseudokinase binds to ATP and autophosphorylates in a metal-independent manner.* Biochem J, 2015. **467**(1): p. 47-62.
11. Hegedus, Z., A. Czibula, and E. Kiss-Toth, *Tribbles: novel regulators of cell function; evolutionary aspects.* Cell Mol Life Sci, 2006. **63**(14): p. 1632-41.
12. Hegedus, Z., A. Czibula, and E. Kiss-Toth, *Tribbles: a family of kinase-like proteins with potent signalling regulatory function.* Cell Signal, 2007. **19**(2): p. 238-50.
13. Murphy, J.M., et al., *A robust methodology to subclassify pseudokinases based on their nucleotide-binding properties.* Biochem J, 2014. **457**(2): p. 323-34.
14. Murphy, J.M., et al., *Molecular Mechanism of CCAAT-Enhancer Binding Protein Recruitment by the TRIB1 Pseudokinase.* Structure, 2015. **23**(11): p. 2111-21.
15. Soubeyrand, S., et al., *TRIB1 Is Regulated Post-Transcriptionally by Proteasomal and Non-Proteasomal Pathways.* PLoS One, 2016. **11**(3): p. e0152346.
16. Wang, J., et al., *Impaired phosphorylation and ubiquitination by p70 S6 kinase (p70S6K) and Smad ubiquitination regulatory factor 1 (Smurf1) promote tribbles homolog 2 (TRIB2) stability and carcinogenic property in liver cancer.* The Journal of biological chemistry, 2013. **288**(47): p. 33667-81.
17. Eyers, P.A., *TRIBBLES: A Twist in the Pseudokinase Tail.* Structure, 2015. **23**(11): p. 1974-6.
18. Uljon, S., et al., *Structural Basis for Substrate Selectivity of the E3 Ligase COP1.* Structure, 2016. **24**(5): p. 687-96.
19. Xu, S., et al., *TRIB2 inhibits Wnt/beta-Catenin/TCF4 signaling through its associated ubiquitin E3 ligases, beta-TrCP, COP1 and Smurf1, in liver cancer cells.* FEBS letters, 2014. **588**(23): p. 4334-41.
20. Mata, J., et al., *Tribbles coordinates mitosis and morphogenesis in Drosophila by regulating string/CDC25 proteolysis.* Cell, 2000. **101**(5): p. 511-22.
21. Seher, T.C. and M. Leptin, *Tribbles, a cell-cycle brake that coordinates proliferation and morphogenesis during Drosophila gastrulation.* Current biology : CB, 2000. **10**(11): p. 623-9.
22. Grosshans, J. and E. Wieschaus, *A genetic link between morphogenesis and cell division during formation of the ventral furrow in Drosophila.* Cell, 2000. **101**(5): p. 523-31.
23. Das, R., et al., *Drosophila tribbles antagonizes insulin signaling-mediated growth and metabolism via interactions with Akt kinase.* PLoS One, 2014. **9**(10): p. e109530.

592 24. Masoner, V., et al., *The kinase domain of Drosophila Tribbles is required for turnover of fly C/EBP*
593 *during cell migration.* Developmental biology, 2013. **375**(1): p. 33-44.
594 25. Liang, K.L., et al., *Human TRIB2 Oscillates during the Cell Cycle and Promotes Ubiquitination and*
595 *Degradation of CDC25C.* International journal of molecular sciences, 2016. **17**(9).
596 26. Salazar, M., et al., *Loss of Tribbles pseudokinase-3 promotes Akt-driven tumorigenesis via FOXO*
597 *inactivation.* Cell death and differentiation, 2015. **22**(1): p. 131-44.
598 27. Zareen, N., S.C. Biswas, and L.A. Greene, *A feed-forward loop involving Trib3, Akt and FoxO*
599 *mediates death of NGF-deprived neurons.* Cell death and differentiation, 2013. **20**(12): p. 1719-
600 30.
601 28. Erazo, T., et al., *The New Antitumor Drug ABTL0812 Inhibits the Akt/mTORC1 Axis by*
602 *Upregulating Tribbles-3 Pseudokinase.* Clinical cancer research : an official journal of the
603 American Association for Cancer Research, 2016. **22**(10): p. 2508-19.
604 29. Du, K., et al., *TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver.* Science,
605 2003. **300**(5625): p. 1574-7.
606 30. Zanella, F., et al., *Human TRIB2 is a repressor of FOXO that contributes to the malignant*
607 *phenotype of melanoma cells.* Oncogene, 2010. **29**(20): p. 2973-82.
608 31. Aime, P., et al., *Trib3 Is Elevated in Parkinson's Disease and Mediates Death in Parkinson's*
609 *Disease Models.* The Journal of neuroscience : the official journal of the Society for
610 Neuroscience, 2015. **35**(30): p. 10731-49.
611 32. Ord, T., et al., *Trib3 is developmentally and nutritionally regulated in the brain but is dispensable*
612 *for spatial memory, fear conditioning and sensing of amino acid-imbalanced diet.* PLoS One,
613 2014. **9**(4): p. e94691.
614 33. Eyers, P.A. and J.M. Murphy, *Dawn of the dead: protein pseudokinases signal new adventures in*
615 *cell biology.* Biochem Soc Trans, 2013. **41**(4): p. 969-74.
616 34. Reiterer, V., P.A. Eyers, and H. Farhan, *Day of the dead: pseudokinases and pseudophosphatases*
617 *in physiology and disease.* Trends Cell Biol, 2014. **24**(9): p. 489-505.
618 35. Zeqiraj, E. and D.M. van Aalten, *Pseudokinases-remnants of evolution or key allosteric*
619 *regulators?* Curr Opin Struct Biol, 2010. **20**(6): p. 772-81.
620 36. Scheeff, E.D., et al., *Structure of the pseudokinase VRK3 reveals a degraded catalytic site, a highly*
621 *conserved kinase fold, and a putative regulatory binding site.* Structure, 2009. **17**(1): p. 128-38.
622 37. Mohanty, S., et al., *Hydrophobic Core Variations Provide a Structural Framework for Tyrosine*
623 *Kinase Evolution and Functional Specialization.* PLoS genetics, 2016. **12**(2): p. e1005885.
624 38. McSkimming, D.I., et al., *ProKinO: a unified resource for mining the cancer kinome.* Human
625 mutation, 2015. **36**(2): p. 175-86.
626 39. Kannan, N., et al., *The hallmark of AGC kinase functional divergence is its C-terminal tail, a cis-*
627 *acting regulatory module.* Proceedings of the National Academy of Sciences of the United States
628 of America, 2007. **104**(4): p. 1272-7.
629 40. Kannan, N. and A.F. Neuwald, *Did protein kinase regulatory mechanisms evolve through*
630 *elaboration of a simple structural component?* Journal of molecular biology, 2005. **351**(5): p. 956-
631 72.
632 41. Oruganty, K., et al., *Identification and classification of small molecule kinases: insights into*
633 *substrate recognition and specificity.* BMC evolutionary biology, 2016. **16**: p. 7.
634 42. Kannan, N. and A.F. Neuwald, *Evolutionary constraints associated with functional specificity of*
635 *the CMGC protein kinases MAPK, CDK, GSK, SRPK, DYRK, and CK2alpha.* Protein science : a
636 publication of the Protein Society, 2004. **13**(8): p. 2059-77.
637 43. Soubeyrand, S., et al., *Role of Tribbles Pseudokinase 1 (TRIB1) in human hepatocyte metabolism.*
638 Biochimica et biophysica acta, 2016. **1862**(2): p. 223-32.
639 44. Wennemers, M., et al., *Regulation of TRIB3 mRNA and protein in breast cancer.* PLoS One, 2012.
640 **7**(11): p. e49439.

641 45. Goldberg, J., A.C. Nairn, and J. Kuriyan, *Structural basis for the autoinhibition of*
642 *calcium/calmodulin-dependent protein kinase I.* Cell, 1996. **84**(6): p. 875-87.
643 46. Yokoyama, T., et al., *Trib1 links the MEK1/ERK pathway in myeloid leukemogenesis.* Blood, 2010.
644 **116**(15): p. 2768-75.
645 47. Guan, H., et al., *Competition between members of the tribbles pseudokinase protein family*
646 *shapes their interactions with mitogen activated protein kinase pathways.* Scientific reports,
647 2016. **6**: p. 32667.
648 48. Jin, G., et al., *Trib1 and Evi1 cooperate with Hoxa and Meis1 in myeloid leukemogenesis.* Blood,
649 2007. **109**(9): p. 3998-4005.
650 49. Dedhia, P.H., et al., *Differential ability of Tribbles family members to promote degradation of*
651 *C/EBP alpha and induce acute myelogenous leukemia.* Blood, 2010. **116**(8): p. 1321-1328.
652 50. Qi, L., et al., *TRB3 links the E3 ubiquitin ligase COP1 to lipid metabolism.* Science, 2006.
653 **312**(5781): p. 1763-6.
654 51. Keeshan, K., et al., *Transformation by Tribbles homolog 2 (Trib2) requires both the Trib2 kinase*
655 *domain and COP1 binding.* Blood, 2010. **116**(23): p. 4948-57.
656 52. Christie, M., et al., *Structure of the PAN3 pseudokinase reveals the basis for interactions with the*
657 *PAN2 deadenylase and the GW182 proteins.* Molecular cell, 2013. **51**(3): p. 360-73.
658 53. Stefely, J.A., et al., *Mitochondrial ADCK3 employs an atypical protein kinase-like fold to enable*
659 *coenzyme Q biosynthesis.* Molecular cell, 2015. **57**(1): p. 83-94.
660 54. Keeshan, K., et al., *Tribbles homolog 2 inactivates C/EBPalpha and causes acute myelogenous*
661 *leukemia.* Cancer Cell, 2006. **10**(5): p. 401-11.
662 55. O'Connor, C., et al., *The presence of C/EBPalpha and its degradation are both required for TRIB2-*
663 *mediated leukaemia.* Oncogene, 2016.
664 56. Grandinetti, K.B., et al., *Overexpression of TRIB2 in human lung cancers contributes to*
665 *tumorigenesis through downregulation of C/EBPalpha.* Oncogene, 2011. **30**(30): p. 3328-35.
666 57. Wang, J., et al., *TRIB2 acts downstream of Wnt/TCF in liver cancer cells to regulate YAP and*
667 *C/EBPalpha function.* Mol Cell, 2013. **51**(2): p. 211-25.
668 58. Qiao, Y., Y. Zhang, and J. Wang, *Ubiquitin E3 ligase SCF(beta-TRCP) regulates TRIB2 stability in*
669 *liver cancer cells.* Biochem Biophys Res Commun, 2013. **441**(3): p. 555-9.
670 59. Liang, K.L., L. Rishi, and K. Keeshan, *Tribbles in acute leukemia.* Blood, 2013. **121**(21): p. 4265-70.
671 60. Rothlisberger, B., et al., *TRIB1 overexpression in acute myeloid leukemia.* Cancer genetics and
672 cytogenetics, 2007. **176**(1): p. 58-60.
673 61. McSkimming, D.I., et al., *KinView: a visual comparative sequence analysis tool for integrated*
674 *kinome research.* Molecular bioSystems, 2016.
675 62. Yokoyama, T., et al., *Identification of TRIB1 R107L gain-of-function mutation in human acute*
676 *megakaryocytic leukemia.* Blood, 2012. **119**(11): p. 2608-11.
677 63. Kitamoto, A., et al., *Association of polymorphisms in GCKR and TRIB1 with nonalcoholic fatty liver*
678 *disease and metabolic syndrome traits.* Endocr J, 2014.
679 64. Zhang, D.E., et al., *Absence of granulocyte colony-stimulating factor signaling and neutrophil*
680 *development in CCAAT enhancer binding protein alpha-deficient mice.* Proc Natl Acad Sci U S A,
681 1997. **94**(2): p. 569-74.
682 65. Qiao, Y., Y. Zhang, and J. Wang, *Ubiquitin E3 ligase SCF(beta-TRCP) regulates TRIB2 stability in*
683 *liver cancer cells.* Biochemical and biophysical research communications, 2013. **441**(3): p. 555-9.
684 66. Wang, J., et al., *TRIB2 acts downstream of Wnt/TCF in liver cancer cells to regulate YAP and*
685 *C/EBPalpha function.* Molecular cell, 2013. **51**(2): p. 211-25.
686 67. Rishi, L., et al., *Regulation of Trib2 by an E2F1-C/EBPalpha feedback loop in AML cell*
687 *proliferation.* Blood, 2014. **123**(15): p. 2389-400.
688 68. Nagel, S., et al., *Activation of Paired-homeobox gene PITX1 by del(5)(q31) in T-cell acute*
689 *lymphoblastic leukemia.* Leuk Lymphoma, 2011. **52**(7): p. 1348-59.

690 69. Ferrando, A.A., et al., *Gene expression signatures define novel oncogenic pathways in T cell acute*
691 *lymphoblastic leukemia.* Cancer Cell, 2002. **1**(1): p. 75-87.
692 70. Sanda, T., et al., *Core transcriptional regulatory circuit controlled by the TAL1 complex in human T*
693 *cell acute lymphoblastic leukemia.* Cancer Cell, 2012. **22**(2): p. 209-21.
694 71. Tan, S.H., et al., *TRIB2 reinforces the oncogenic transcriptional program controlled by the TAL1*
695 *complex in T-cell acute lymphoblastic leukemia.* Leukemia, 2016. **30**(4): p. 959-62.
696 72. Argiropoulos, B., et al., *Linkage of Meis1 leukemogenic activity to multiple downstream effectors*
697 *including Trib2 and Ccl3.* Exp Hematol, 2008. **36**(7): p. 845-59.
698 73. Keeshan, K., et al., *Tribbles homolog 2 (Trib2) and HoxA9 cooperate to accelerate acute*
699 *myelogenous leukemia.* Blood Cells Mol Dis, 2008. **40**(1): p. 119-21.
700 74. Keeshan, K., et al., *Co-operative leukemogenesis in acute myeloid leukemia and acute*
701 *promyelocytic leukemia reveals C/EBPalpha as a common target of TRIB1 and PML/RARA.*
702 Haematologica, 2016. **101**(10): p. 1228-1236.
703 75. Aster, J.C., et al., *Essential roles for ankyrin repeat and transactivation domains in induction of T-*
704 *cell leukemia by notch1.* Molecular and cellular biology, 2000. **20**(20): p. 7505-15.
705 76. Wouters, B.J., et al., *Distinct gene expression profiles of acute myeloid/T-lymphoid leukemia with*
706 *silenced CEBPA and mutations in NOTCH1.* Blood, 2007. **110**(10): p. 3706-14.
707 77. Liang, K.L., et al., *TRIB2 regulates normal and stress-induced thymocyte proliferation.* Cell
708 discovery, 2016. **2**: p. 15050.
709 78. Stein, S.J., et al., *Trib2 Suppresses Tumor Initiation in Notch-Driven T-ALL.* PLoS One, 2016. **11**(5):
710 p. e0155408.
711 79. Salome, M., J. Campos, and K. Keeshan, *TRIB2 and the ubiquitin proteasome system in cancer.*
712 Biochemical Society transactions, 2015. **43**(5): p. 1089-94.
713 80. Hill, R., et al., *TRIB2 as a biomarker for diagnosis and progression of melanoma.* Carcinogenesis,
714 2015. **36**(4): p. 469-77.
715 81. Lin, Z.Y., et al., *MicroRNA-224 inhibits progression of human prostate cancer by downregulating*
716 *TRIB1.* Int J Cancer, 2014. **135**(3): p. 541-50.
717 82. Tang, B., et al., *Inhibition of tribbles protein-1 attenuates radioresistance in human glioma cells.*
718 Sci Rep, 2015. **5**: p. 15961.
719 83. Bhushan, L. and R.P. Kandpal, *EphB6 receptor modulates micro RNA profile of breast carcinoma*
720 *cells.* PLoS One, 2011. **6**(7): p. e22484.
721 84. Puiffe, M.L., et al., *Characterization of ovarian cancer ascites on cell invasion, proliferation,*
722 *spheroid formation, and gene expression in an in vitro model of epithelial ovarian cancer.*
723 Neoplasia, 2007. **9**(10): p. 820-9.
724 85. Puskas, L.G., et al., *Gene profiling identifies genes specific for well-differentiated epithelial thyroid*
725 *tumors.* Cell Mol Biol (Noisy-le-grand), 2005. **51**(2): p. 177-86.
726 86. Miyoshi, N., et al., *Abnormal expression of TRIB3 in colorectal cancer: a novel marker for*
727 *prognosis.* Br J Cancer, 2009. **101**(10): p. 1664-70.
728 87. Wennemers, M., et al., *TRIB3 protein denotes a good prognosis in breast cancer patients and is*
729 *associated with hypoxia sensitivity.* Radiother Oncol, 2011. **101**(1): p. 198-202.
730 88. Wennemers, M., et al., *Tribbles homolog 3 denotes a poor prognosis in breast cancer and is*
731 *involved in hypoxia response.* Breast Cancer Res, 2011. **13**(4): p. R82.
732 89. Zhou, H., et al., *Knockdown of TRB3 induces apoptosis in human lung adenocarcinoma cells*
733 *through regulation of Notch 1 expression.* Mol Med Rep, 2013. **8**(1): p. 47-52.
734 90. Todd, A.E., C.A. Orengo, and J.M. Thornton, *Sequence and structural differences between enzyme*
735 *and nonenzyme homologs.* Structure, 2002. **10**(10): p. 1435-51.
736 91. Todd, A.E., C.A. Orengo, and J.M. Thornton, *Plasticity of enzyme active sites.* Trends in
737 biochemical sciences, 2002. **27**(8): p. 419-26.

738 92. Eyers, P. A. and J. M. Murphy, The Evolving world of pseudoenzymes: Proteins, prejudice and
739 zombies. BMC Biology 2016. *In Press*
740 93. Bailey, F.P., et al., *Going for broke: targeting the human cancer pseudokinome.* The Biochemical
741 journal, 2015. **465**(2): p. 195-211.
742 94. Foulkes, D.M., et al., *Tribbles pseudokinases: novel targets for chemical biology and drug*
743 *discovery?* Biochemical Society transactions, 2015. **43**(5): p. 1095-103.
744 95. Schug, J., *Using TESS to predict transcription factor binding sites in DNA sequence.* Current
745 protocols in bioinformatics / editoral board, Andreas D. Baxevanis ... [et al.], 2008. **Chapter 2**:
746 p.Unit 2 6.
747 96. Neuwald, A.F., *Rapid detection, classification and accurate alignment of up to a million or more*
748 *related protein sequences.* Bioinformatics, 2009. **25**(15): p. 1869-75.
749 97. Neuwald, A.F., *Bayesian classification of residues associated with protein functional divergence:*
750 *Arf and Arf-like GTPases.* Biology direct, 2010. **5**: p. 66.
751 98. Nguyen, T., et al., *Co-conserved MAPK features couple D-domain docking groove to distal*
752 *allosteric sites via the C-terminal flanking tail.* PLoS One, 2015. **10**(3): p. e0119636.

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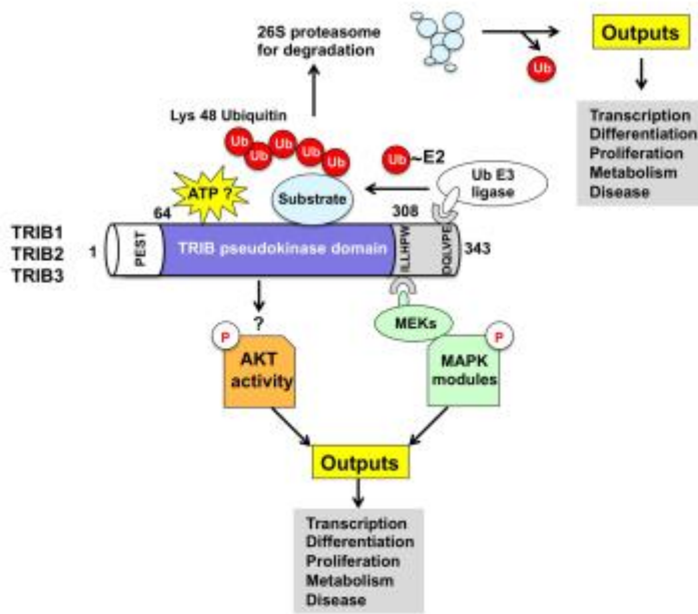

757 Figure 1 Eyers, Keeshan and Kannan

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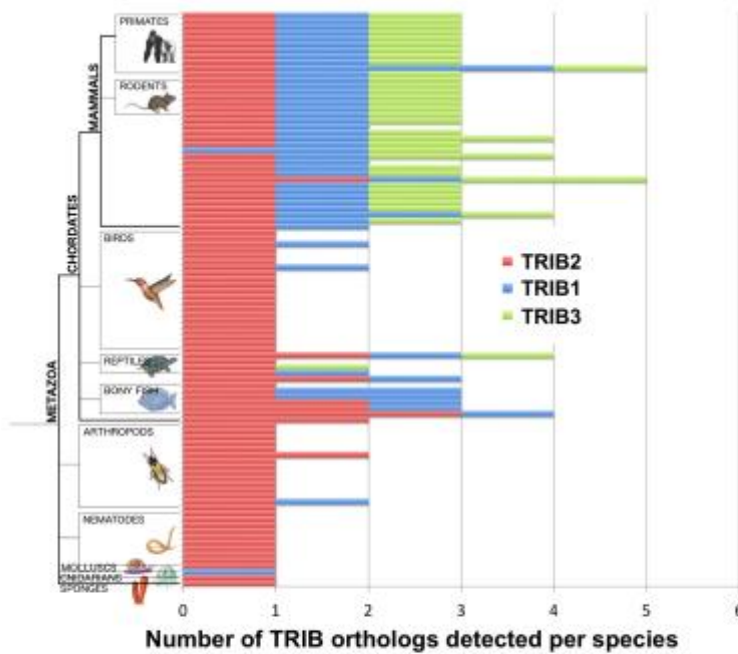

759 Figure 2 Eyers, Keeshan and Kannan

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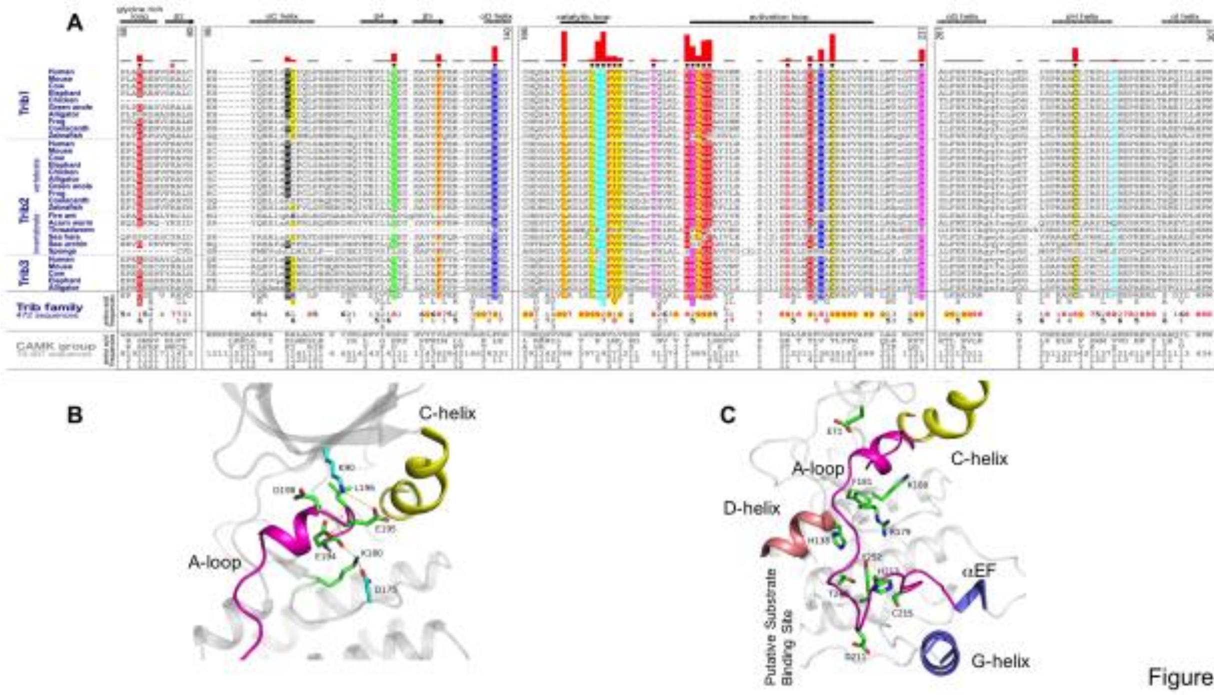

Figure 3

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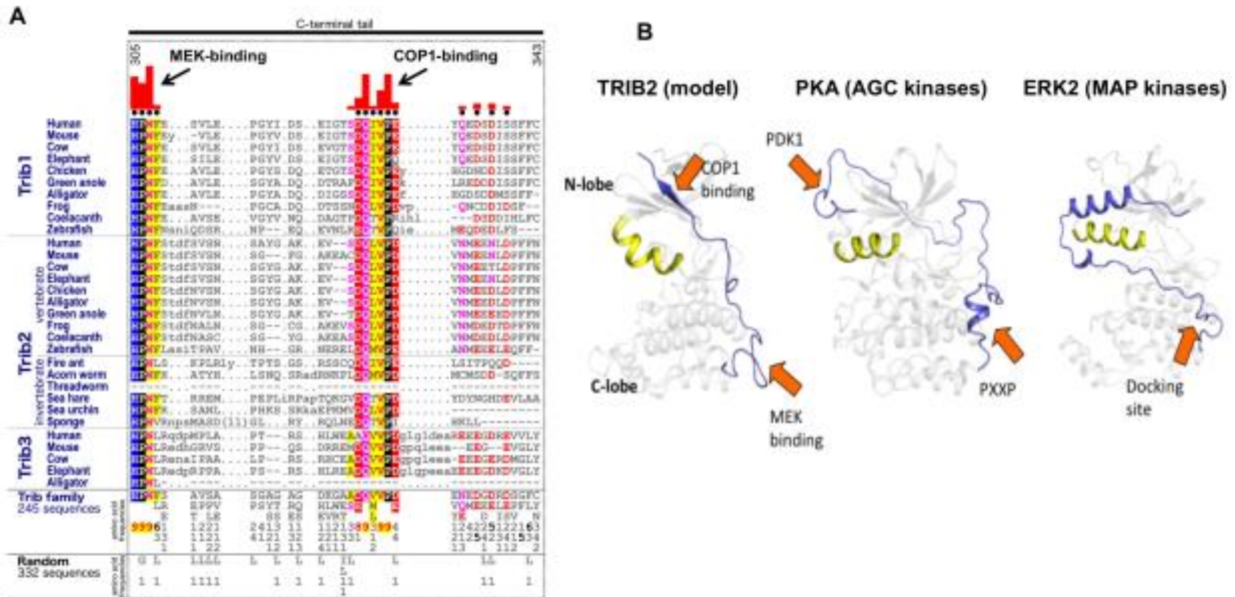

Figure 4 Eyers, Keeshan and Kannan

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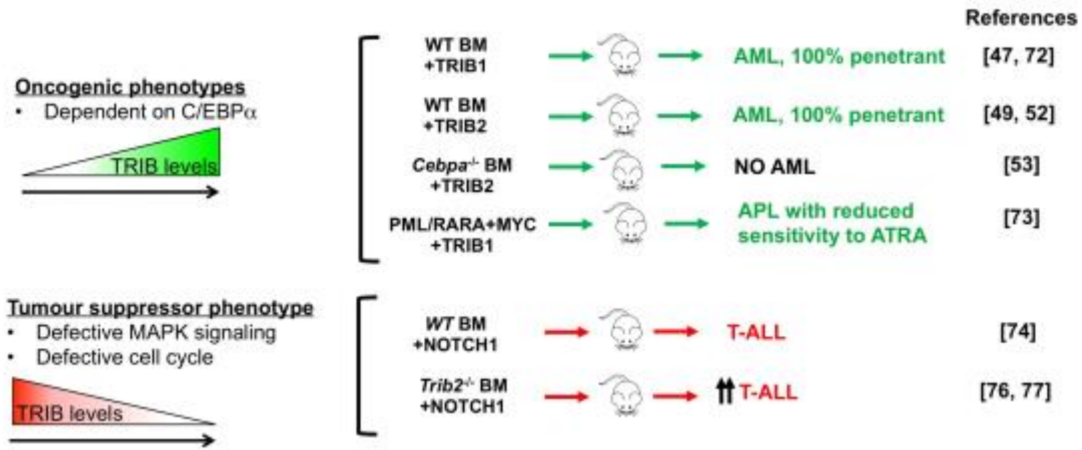

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Figure 5 Eyers, Keeshan and Kannan

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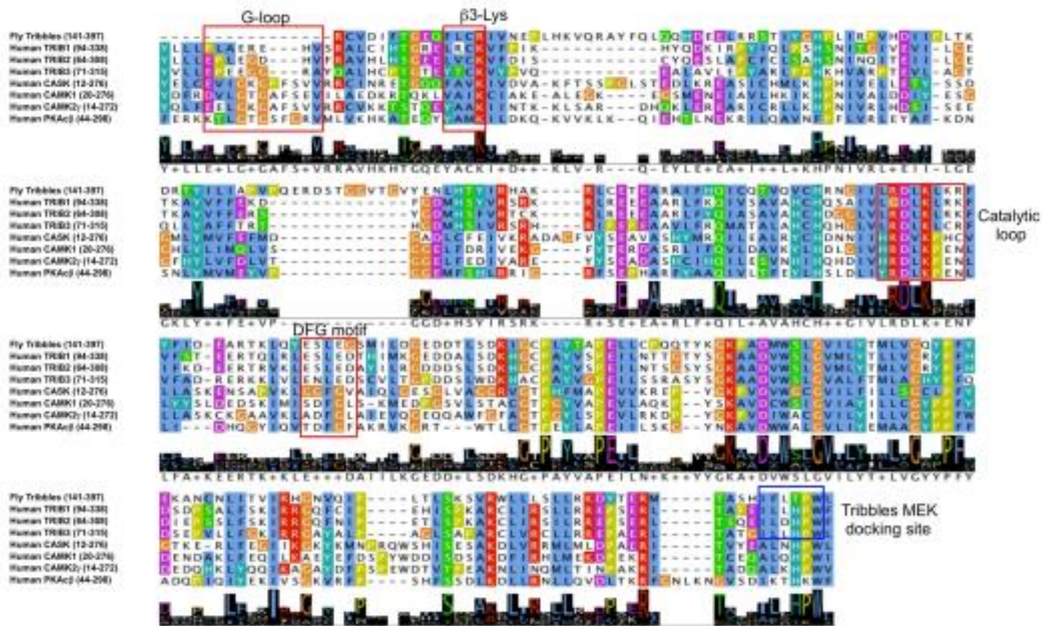

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Box 1 Eyers, Keeshan and Kannan

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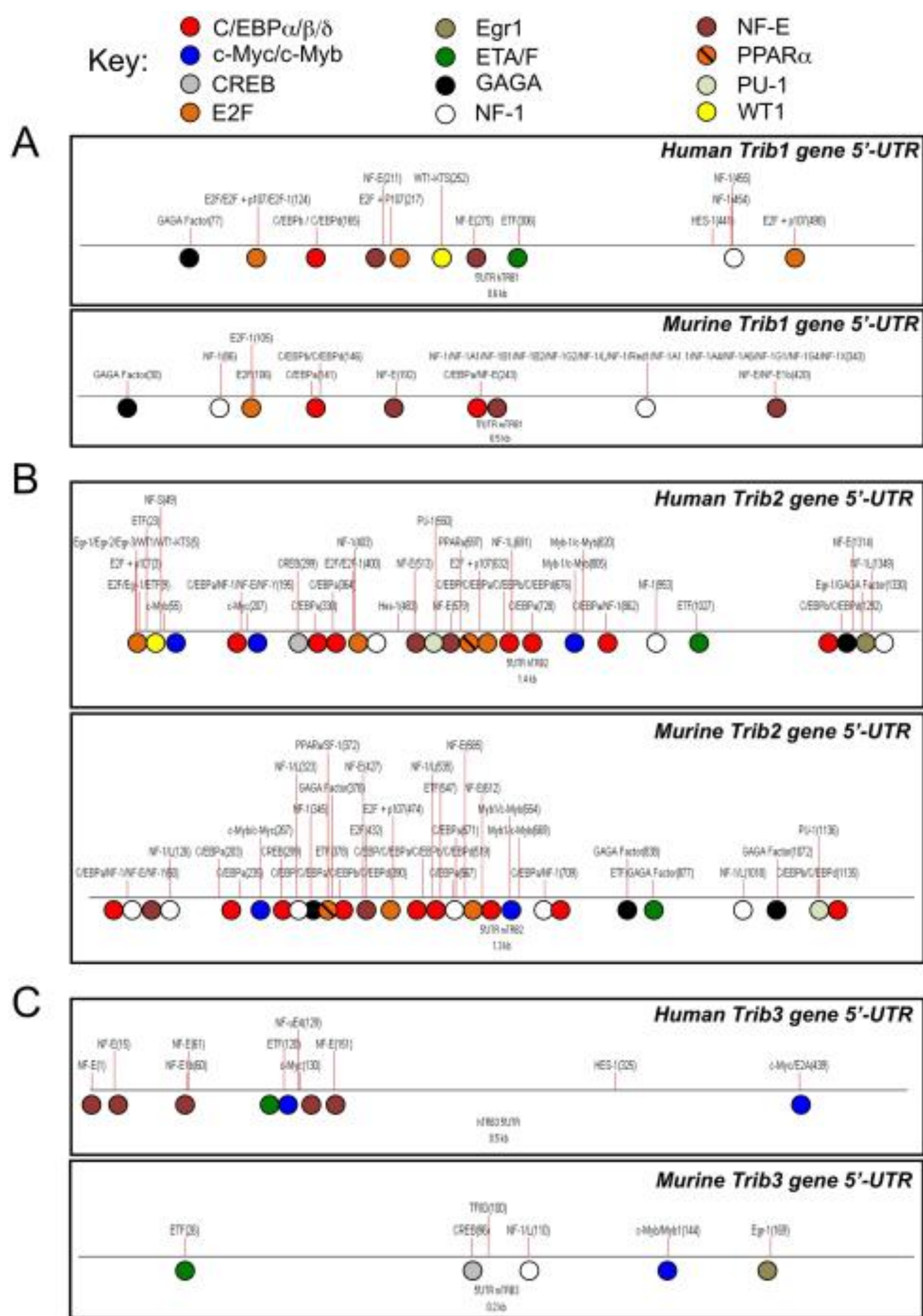

Box 2 Eyers, Keeshan and Kannan

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772 Trends Box:

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774 **Tribbles in the 21st Century: The evolving role of Tribbles**

775 **pseudokinases in biology and disease**

776

777 Patrick A Eyers, Karen Keeshan and Natarajan Kannan

778

- 779 Pseudoenzymes are inactive counterparts of classical enzymes and have evolved in all 780 kingdoms of life, where they regulate a vast array of biological processes. The 781 pseudokinases are one of the best-studied families of human pseudoenzyme.

782

- 783 Eukaryotic Tribbles pseudokinases evolved from a common ancestor (the human TRIB2 784 homolog), and contain a highly atypical pseudokinase domain fused to a unique docking 785 site in an extended C-tail that binds to ubiquitin E3 ligases.

786

- 787 Tribbles evolution has led to the appearance of three mammalian TRIB pseudokinases, 788 termed TRIB1, TRIB2 and TRIB3, which contain both unique and shared features.

789

- 790 In cells, Tribbles pseudokinases act as modulators of substrate ubiquitination and as 791 molecular scaffolds for assembly and regulation of signaling modules, including the 792 C/EBPα transcription factor and AKT and ERK networks.

793

- 794 TRIB1 and TRIB2 possess potent oncogenic activities in vertebrate cells, and recent 795 evidence also suggests that TRIB2 can act as a tumour suppressor, consistent with the 796 requirement for balanced TRIB signaling in the regulation of transcription, differentiation, 797 proliferation and apoptosis.

798

799 Outstanding Questions Box:

800

801 **Tribbles in the 21st Century: The evolving role of Tribbles**

802 **pseudokinases in biology and disease**

803

804 Patrick A Eyers, Karen Keeshan and Natarajan Kannan

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807 • What is the structural basis for the distinct cellular roles of TRIB pseudokinases. In
808 particular, how do subtle variations in sequence identified in the three distinct, but
809 related, pseudokinase domains drive cell signaling. Will a combination of X-ray and NMR
810 approaches be needed to evaluate TRIB dynamics in the complete polypeptide in
811 comparison to the isolated pseudokinase domain?
812

813 • What are the specific binding partners of the three TRIB pseudokinase polypeptides,
814 within and across the PEST region, the pseudokinase domain and the C-tail? Can these
815 regions be trapped with substrates bound and studied by Mass Spectrometry, and are all
816 substrates that bind actively ubiquitinated? Furthermore, is this process controlled on the
817 pseudokinase, or in a processive manner after release? Finally, how many different
818 ubiquitin E3 ligases do the TRIB pseudokinases engage?
819

820 • What is the role (if any) of the vestigial ATP binding detected in human TRIB2 and TRIB3
821 *in vitro*. This is an important question, since the concentration of ATP in cells is in the mM
822 range, which might be sufficient for TRIB pseudokinases to possess nucleotide-
823 dependent mechanisms, as appears to be the case for several other pseudokinases. In
824 this context, do TRIB pseudokinases undergo switching mechanisms that change the
825 accessibility of the highly atypical nucleotide-binding site, coupling it to ubiquitination?
826

827 • Leading on from this, is the atypical nucleotide-binding site suitable for targeting with
828 small molecules? Do known protein kinase inhibitors have unappreciated ‘off-target’
829 effects on TRIB pseudokinases in cells? Can small molecules be designed to probe TRIB
830 structural dynamics and cell biology? In particular, can compounds be identified that
831 promote changes in TRIB stability in different cell types. These classes of chemical might
832 be very useful leads for new types of drugs
833

834 • How are the expression levels of TRIB pseudokinases regulated under physiological
835 conditions, and prior to and during pathology? Related to this, what are the
836 transcriptional networks in operation that fulfil the obligations of the TRIB proteins in
837 eukaryotes, and how have these changed during evolution?
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