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Kerk and Moorhead: A phylogenetic survey of myotubularin genes of eukaryotes: distribution, protein structure, evolution, and gene expression. BMC Evolutionary Biology 2010 10:196.
http://hdl.handle.net/1880/48140
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A phylogenetic survey of myotubularin genes of eukaryotes: distribution, protein structure, evolution, and gene expression

David Kerk, Greg BG Moorhead*

Abstract

Background: Phosphorylated phosphatidylinositol (PtdIns) lipids, produced and modified by PtdIns kinases and phosphatases, are critical to the regulation of diverse cellular functions. The myotubularin PtdIns-phosphate phosphatases have been well characterized in yeast and especially animals, where multiple isoforms, both catalytically active and inactive, occur. Myotubularin mutations bring about disruption of cellular membrane trafficking, and in humans, disease. Previous studies have suggested that myotubularins are widely distributed amongst eukaryotes, but key evolutionary questions concerning the origin of different myotubularin isoforms remain unanswered, and little is known about the function of these proteins in most organisms.

Results: We have identified 80 myotubularin homologues amidst the completely sequenced genomes of 30 organisms spanning four eukaryotic supergroups. We have mapped domain architecture, and inferred evolutionary histories. We have documented an expansion in the Amoebozoa of a family of inactive myotubularins with a novel domain architecture, which we dub "IMLRK" (inactive myotubularin/LRR/ROCO/kinase). There is an especially large myotubularin gene family in the pathogen Entamoeba histolytica, the majority of them IMLRK proteins. We have analyzed published patterns of gene expression in this organism which indicate that myotubularins may be important to critical life cycle stage transitions and host infection.

Conclusions: This study presents an overall framework of eukaryotic myotubularin gene evolution. Inactive myotubularin homologues with distinct domain architectures appear to have arisen on three separate occasions in different eukaryotic lineages. The large and distinctive set of myotubularin genes found in an important pathogen species suggest that in this organism myotubularins might present important new targets for basic research and perhaps novel therapeutic strategies.

Background

Phosphatidylinositol (PtdIns) phospholipids are quantitatively minor but functionally significant membrane lipid components which have been shown to be involved in regulating diverse aspects of cellular function, such as proliferation, survival, growth, cytoskeletal reorganization, and various membrane trafficking events. The inositol ring can be phosphorylated at the D3, D4 or D5 position to produce a set of seven distinct phosphorylated derivatives, which are preferentially located in various cellular membranes or microdomains, specifying their identity, and mediating cellular functions by recruiting various effector proteins with specialized lipid-binding domains [1]. The homeostasis of these phosphorylated PtdIns lipids is mediated by a number of specific kinases and phosphatases.

Myotubularins are members of the protein tyrosine phosphatase (PTP) superfamily, which feature a characteristic HCX(5)R catalytic motif, where the cysteine is the catalytic residue, the histidine is important for the nucleophilic properties of the cysteine, and the arginine is important in coordinating the substrate phosphate group. Myotubularins have been shown to be specific lipid phosphatases, cleaving the D3 phosphate from PtdIns3P and PtdIns(3,5)P2. There is a large myotubularin family in humans (14 genes) which encode both catalytically active and inactive members. Mutations in
either active or inactive members of this family bring about human disease, which involves chiefly skeletal muscle (X-linked myotubular myopathy [XLMTM]) or peripheral neurons (Charcot-Marie-Tooth [CMT] neuropathies) [2-4]. Previous phylogenetic studies have reported the presence of myotubularin genes in plants, fungi and some protists, with the latter group only containing both active and inactive forms [2,5].

This study presents a systematic survey of myotubularin genes in a large number of completely sequenced eukaryotic genomes, representing a broad array of taxonomic groups. Most genomes contain one to a few myotubularin genes, though they are absent in certain groups. The evidence is consistent with the independent appearance of inactive myotubularin genes, featuring novel domain combinations, in different taxonomic groups. The greatest expansion of the myotubularin gene family yet observed occurs in the pathogenic species *Entamoeba histolytica*. Functional evidence derived from published gene expression studies indicates that these genes may be important in pathogen transmission and host infection.

**Results**

**Phylogenetic Distribution, Gene Evolution, Domain Architecture**

Recent work in eukaryotic systematics has increasingly defined large organismal “supergroups” encompassing many traditional smaller groups [6-8]. We have conducted a broad survey of fully sequenced genomes amongst these large organismal groups for the presence of myotubularin gene homologues. Only the Rhizaria were excluded as there is as yet no completed genome in that group. Our results are summarized in Figure 1. We searched 30 genomes, and identified 80 sequences.

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**Figure 1 Myotubularin Homologues in Eukaryotic Protein Datasets**

Listed is the set of eukaryotic species with completely sequenced genomes whose protein datasets were searched for myotubularin homologues. The number of myotubularin homologues detected for each genome is listed. Color coding: Excavates (gray); Unikonts, Amoebozoa (pale blue); Unikonts, Choanoflagellates/Metazoa (medium blue); Unikonts, Fungi (dark blue); Chromalveolates (yellow); Plantae (green). Taxonomy was taken from: NCBI Taxonomy Browser [http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Root; Tree of Life [http://tolweb.org/Eukaryotes/3]; and Koonin, 2010 [8]. The URLs for downloading of all organismal datasets, and the original publication references, are given in Additional File 4. Figure design after Gazave et al., 2009 [68].

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found that myotubularin genes are nearly ubiquitous in eukaryotes, being readily identifiable in all the major eukaryotic groups and in all genomes examined with the notable exception of the obligate intracellular parasites *Encephalitozoon cuniculi* (Microsporidia) and *Plasmodium falciparum* (Apicomplexa) and eukaryotic algae, both red (*Cyanidioschyzon merolae*) and green (*Ostreococcus sp.*, *Chlamydomonas reinhardtii*). Most organisms (19 out of 24 species with myotubularins) posses one to three genes. The notable exception to this general pattern occurs in members of the Unikonta (Amoebozoa, Chao-

*G[((57x470)larin proteins contain both amyotubularin phosphatase presented in Figure 2. It is apparent that nearly all myotub-

myotubularin gene encoded proteins. The results are pre-

Methods to determine the molecular architecture of phos-

domain and a PH-GRAM domain ([10](#)). The nearly constant presence of the PH-

domain ([10](#)) suggests that the PH-GRAM domain is a standard feature of these pro-

PH-GRAM domain sequences can be very divergent, but clearly recognizable. The lack of a PH-GRAM domain, unique to these Excavate inactive myotubularins, suggests that they comprise a single gene lineage. *Amoebozoan IMLRK (inactive Myotubularin/LRR/ROCO/ Kinase) Genes and Proteins*

The amoebozoans *Dictyostelium* and *Entamoeba* each have a large number of myotubularin homologues (see Figure 1 and Figure 2). *Dictyostelium* has nine active myotubularin subunits, and *Entamoeba* has eight. In addition, there are a number of inactive myotubularin subunits. The *Dictyostelium* gene pat1 (encoding sequence DDB0191503) was previously incorrectly reported to contain an active myotubularin domain [12]. In addition, this protein contains a LRR domain, a recently described ROCO domain [13,14] (comprised of a ROC [Ras of complex proteins] and COR [C-terminal of ROC] region), and a protein kinase domain. The LRR/ROCO/kinase architecture was also known to be shared by *Dictyostelium* sequence DDB0191512, which also has an N-terminal Rho-GAP domain. By use of a sensitive myotubularin-sequence based HMM search strategy, we found that this sequence also contains an inactive myotubularin domain. Further application of this HMM search revealed that *Entamoeba* contains eleven proteins with divergent, but clearly recognizable inactive myotubularin domains (EH1_140980, EH1_137960, EH1_185230, EH1_048230, EH1_151670, EH1_107230, EH1_135010, EH1_141820, EH1_078170, EH1_197200, EH1_188050). Our findings confirm and extend previous observations [5]. Further examination of the domain architecture of the newly discovered *Enta-

amoeba* inactive myotubularin sequences revealed that 9 of them also showed significant similarity to the solved structures of LRR proteins and protein kinases, and weaker but still significant similarity to the solved struc-

ture of a bacterial ROCO protein (PDB: 3dpu_A) [15] as detected by both the FFAS03 (Fold and Function

The catalytic loop signature of human myotubularins is: HCSDGWDR [2]. Inspection of the myotubularin sequence alignment presented in Figure 3 shows that this is found invariant in most of the myotubularin sequences, indicating that they all share a common local active site architecture and catalytic mechanism. One of the notable features of human myotubularins is the presence of several catalytically inactive subunits, resulting from mutations to the key catalytic cysteine and argi-
nine residues in the catalytic loop region. It has been previously noted that myotubularin genes with apparently inactive catalytic loop signatures can be observed in *Giardia* and *Dictyostelium*, suggesting that inactive subunits arose early in evolution [2,5]. Our work confirms these findings, and sheds further light on the origin of these sequences. Three Excavate myotubularin sequences lack a PH-GRAM domain, the only sequences we observed with this characteristic (see Figure 2). *Giardia* sequence GL50803_112811 lacks both the cysteine and arginine residues from the catalytic loop region (see Figure 3). *Leishmania* sequence LmjF12.0320 and *Trypanosoma* sequence Tb927.6.870 each possess both the cysteine and the arginine, but lack the histidine preceding the cysteine. Since this histidine is universally conserved in active PTP phosphatases, and has been shown to be important in the catalytic mechanism by altering the nucleophilic properties of the neighboring cysteine [11], it is likely that these proteins are also catalytically inactive. The lack of a PH-GRAM domain, unique to these Excavate inactive myotubularins, suggests that they comprise a single gene lineage.
Candidate myotubularin homologue sequences were obtained by searching the protein datasets of fully sequenced eukaryotic genomes, as detailed in Methods. PH-GRAM, myotubularin phosphatase, and other structural domains were identified as detailed in Methods. To conserve space, organisms are identified in the Figure as genus names only. Organisms are grouped in this figure by taxonomic category as in Figure 1. The domain architecture of human MTMR2 is given for orientation purposes. The organisms are as follows: Homo sapiens, Giardia lamblia, Leishmania major, Trypanosoma brucei, Dictyostelium discoideum, Entamoeba histolytica, Monosiga brevicollis, Trichoplax adhaerens, Nematostella vectensis, Aspergillus niger, Fusarium graminearum, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Laccaria bicolor, Ustilago maydis, Cryptosporidium hominis, Paramecium tetraurelia, Tetrahymena thermophila, Thalassiosira pseudonana, Arabidopsis thaliana, Oryza sativa, Populus trichocarpa, Sorghum bicolor, Vitis vinifera, Physcomitrella patens. The complete set of sequences illustrated in the Figure, along with database accession numbers, is available as Additional File 5. As detailed in Methods, some incomplete candidate myotubularin sequences with annotation mistakes were corrected with additional sequence, and are denoted with the suffix "C" in the Figure. Sequence "Mbrevi5R3" was assembled manually from individual genomic sequence reads uncincorporated into scaffolds, through use of TBLASTN against Monosiga genomic DNA with Nematostella myotubularin homologue query sequences.
Figure 3 Alignment of Catalytic Region of Myotubularin Domain Sequences. Human myotubularin sequences were obtained from the literature and database keyword searches. For the other species represented, candidate myotubularin sequences were obtained by either utilizing BLASTP at NCBI [69] or searching the protein datasets of fully sequenced eukaryotic genomes, as detailed in Methods. The contiguous sequence encompassing the PH-GRAM domain and the myotubularin phosphatase domain was identified as detailed in Methods, and multiple alignment was performed as detailed in Methods. Sequences are presented in the general order of taxonomic groups specified in Figure 1, with the exception that Choanoflagellate/Metazoan sequences are grouped according to phylogenetic classification as in Table 2. Non-human sequences in the alignment are generally designated by an organism prefix followed by numerals. This Figure presents the portion of the alignment around the catalytic loop region. The full alignment is presented as Additional File 1. At the top of the alignment, in the line designated "SS", is depicted the secondary structure of the solved structure of human MTMR2 (PDB:1LW3[70]). "H" indicates alpha helix, "C" indicates random coil, "E" indicates beta strand. Above the alignment a blue bar (positions 1380 - 1389) indicates the location of the catalytic motif of the myotubularin phosphatase domain (HCSDGWDR for active subunits). The sequences for all myotubularin homologues, along with their database accession numbers, and designations used in this Figure, are presented in Additional File 5. The URLs for all web sites used to obtain organism specific databases, plus original literature citations, are presented in Additional File 4.
 Assignment System ) sequence:profile technique, and the HHIPred (HMM-HMM [Hidden Markov Model] structure prediction) profile:profile technique. This indicated that these nine proteins might also share the inactive myotubularin/LRR/ROCO/kinase architecture previously detected in Dictostelium sequences. We suggest the acronym “IMLRK” to refer to this somewhat cumbersome domain architecture.

To confirm the identity of the ROCO domains of these Entamoeba sequences we performed iterative multiple sequence alignments, HMM construction, database searches and realignment, to assemble the data presented as Figure 4. During this process, we identified several previously unreported ROCO proteins (2 from Monosiga and 9 from Trichoplax). The alignment presents a comparison between our set of newly identified ROCO domain sequences and those from previously characterized Dictostelium proteins. In their report of the solved structure of a bacterial ROCO protein, Gotthardt et al. [15] identified residues important to both the function of the bacterial protein, and animal ROCO protein homologues. These include residues in the ROC domain important for GTPase binding and residues in both the ROC and COR domains important for domain interactions and GTPase activity (see Legend to Figure 4). It is evident by inspection of the alignment in Figure 4 that on the whole, conservation of this critical residue set for the Entamoeba IMLRK sequences is poor. Despite the overall apparent similarity of these sequences to the rest of the comparison set, several of the Entamoeba sequences have deletions in these critical residues, and would therefore presumably lack GTPase activity. Only one Entamoeba sequence (EHI_048230) has a set of residues which might confer enzymatic activity.

Comparison with sequence models at NCBI CDD (Conserved Domain Database) indicates that the protein kinase domains of the Entamoeba IMLRK proteins resemble both Ser-Thr and Tyr kinases (see Table 1). This is consistent with previous characterization of kinase domains in ROCO proteins as being of the TKL (Tyrosine kinase-like) group [16,17]. We performed a multiple sequence alignment with these kinase domains, which is presented in Figure 5. We examined the group of ten important functional sequence positions characterized by Kannan et al. [18]. For 7 of the 9 Entamoeba sequences, all of these critical functional residues are conserved. The exceptions are: EHI_107230, where there is an H to T mutation at the position corresponding to PKA (Protein Kinase A) H158; and EHI_135010, where there is a D to N mutation at the position corresponding to PKA D166, and an N to A mutation at the position corresponding to PKA N171. Thus we would predict that nearly all of these sequences are catalytically active [18].

Five of the newly discovered Entamoeba sequences have predicted N-terminal Rho-GAP (Rho-GTPase Activating Protein) domains. Of these five domains, however, only one (that for EHI_048230) is strong enough to appear in a domain search at NCBI CDD with default settings, indicating that it is probably enzymatically active. The enzymatic activity of the other four domains is questionable, due to their evident sequence divergence. These five sequences with an N-term Rho-GAP domain resemble the architecture of the Dictostelium gene roco9 (protein sequence DDB0191512), and it is possible that they represent a distinct gene lineage.

The myotubularin domains of the IMLRK proteins are divergent, as is evident by inspection of our sequence alignments (Figure 3 and Additional File 1). The Entamoeba IMLRK proteins have all suffered deletion of the α14 region of the phosphatase domain (positions 1701 - 1706 of our reference alignment [Additional File 1]). Sequence EHI_197200 is clearly the most divergent of the group. It is also missing the α8 and α9 regions, and the C-terminus of the phosphatase domain (from α14 on). In summary, the IMLRK domain architecture is distinctive, being seen in no other taxonomic group besides the Amoebozoa, which suggests that the origin of these genes comprises a second, independent event in myotubularin gene evolution.

Finally, we attempted to determine, by multiple sequence alignment and phylogenetic tree analysis (data not shown) the possible origin of the two inactive Entamoeba myotubularins without a ROCO domain. EHI_188050 appears to be closely related to an active subunit (EHI_104710), and has therefore probably recently suffered inactivating mutations. The origin of EHI_140980 is more obscure - it does not appear to be closely related to any of the other inactive Entamoeba myotubularins.

Myotubularins in the Choanoflagellate/Metazoan Assemblage

Previous phylogenetic analyses [2,3] of myotubularin sequences from human, other vertebrates, and a collection of invertebrates defined six similarity clusters - three composed of catalytically active subunits (in human: [‘M1 Group’: MTM1 {Myotubular myopathy}, MTM1 {MTM-related}, MTM2]; [‘R3 Group’: MTM3, MTM4]; [‘R6 Group’: MTMR6, MTMR7, MTMR8]) and three composed of catalytically inactive subunits (in human: [‘R5 Group’: MTMR5, MTMR13]; [‘R9 Group’: [‘R10 Group’: MTMR10, MTMR11, MTMR12]). We have extended this analysis by finding previously unreported myotubularin homologues in the metazoaans Nematostella and Trichoplax, and the choanoflagellate Monosiga. Our results are presented in Table 2, along with Bayesian and Maximum Likelihood clade supports for each group. Bayesian support is high for all groups, with the mean posterior probability exceeding...
Figure 4 Alignment of ROCO Domain Sequences. ROCO domain sequences were identified and aligned as detailed in Methods. The alignment presents the sequences of six ROCO domain reference proteins: "Ct_ROCO" (Chlorobium tepidum); "Ns_LRRP1" (Nostoc sp. PCC 7120); "Mb_ROCO1" (Methanosarcina barkeri str. Fusaro); "Hs_LRK1" (Homo sapiens); "Hs_LRRK2" (Homo sapiens); and "Ce_LRK1" (Caenorhabditis elegans). Most of the other sequences are designated by an organism prefix, followed by a number from the appropriate organism-specific protein database. Several Dictyostelium ("Dd") sequences are referred to by their gene names. Further information about the reference and candidate ROCO sequences, including organism prefixes and database accession numbers, are provided in Additional File 6. Conserved residues shown by Gotthardt et al. [15] as being critical to the functioning of both the bacterial protein and the human ROCO protein homologue LRRK2 are marked with blue lettering outlining and blue arrows. These positions are as follows: "T484" (our T53); "L487" (our L61); "G518" (our G110); and "Y804" (our Y642). Above the alignment is shown in red the secondary structure of the solved structure of the ROCO domain of Chlorobium tepidum (PDB: 3dpu_A). "A" indicates alpha helix, "B" indicates beta strand, and arrowhead symbols ("<" and ">") denote the beginning and ending of secondary structure regions. The functionally important "Switch I" ("SW1") and "Switch II" ("SW2") regions are indicated. Areas with "+++" symbols in purple represent poorly aligned sequence regions which have been edited from the alignment. The initial sequence region (positions 1-365) represents the ROC domain. The beginning of the COR domain is indicated.
The myotubularins of the PH-GRAM domain (predicted to be active or inactive) consistent with their class placement. The myotubularins of the PH-GRAM domain, and a myotubularin phosphatase domain. This is true for the new sequences Tad51481 (Trichoplax) and Nv19357 (Nematostella). However sequence M001750622 (Monosiga) has an additional domain of unknown function at the extreme N-terminus, and lacks the C-terminal PH domain. This may indicate that the stable “R5” subunit domain architecture had not yet been achieved at this early stage of myotubulin gene evolution. Myotubulin representatives can only be identified clearly for the R5, R6, and R9 groups. This would suggest that the split between catalytically active and inactive myotubulin groups had been completed at the very base of the Metazoan clade.

The situation is less clear for the Monosiga genome. Representatives can only be identified clearly for the R5, R6, and R9 groups. This would suggest that the split between catalytically active and inactive myotubulin representatives characteristic of the Metazoan clade had occurred.

### Table 1 ROCO Kinase Domain CDD Hits

| Query Sequence | NCBI CDD Hits | E values |
|----------------|---------------|----------|
| EHI_157960     | cd00180, S_TKc | 4.00E-35 |
|                | cd00192, PTKc  | 2.00E-32 |
| EHI_151670     | cd00192, PTKc  | 8.00E-38 |
|                | cd00180, S_TKc | 7.00E-28 |
| EHI_155010     | cd00192, PTKc  | 5.00E-33 |
|                | cd00180, S_TKc | 7.00E-28 |
| EHI_048230     | cd00180, S_TKc | 1.00E-28 |
|                | cd00192, PTKc  | 5.00E-26 |
| EHI_107230     | cd00180, S_TKc | 1.00E-35 |
|                | cd00180, S_TKc | 1.00E-31 |
| EHI_185230     | cd00180, S_TKc | 8.00E-34 |
|                | cd00192, PTKc  | 3.00E-32 |
| EHI_078170     | cd00192, PTKc  | 7.00E-37 |
|                | cd00180, S_TKc | 3.00E-31 |
| EHI_141820     | cd00192, PTKc  | 1.00E-45 |
|                | cd00180, S_TKc | 3.00E-41 |
| EHI_197200     | cd00192, PTKc  | 2.00E-35 |
|                | cd00180, S_TKc | 3.00E-31 |
| DDB0191503     | cd00192, PTKc  | 2.00E-52 |
|                | cd00180, S_TKc | 1.00E-38 |
| DDB0191512     | cd00192, PTKc  | 2.00E-52 |
|                | cd00180, S_TKc | 3.00E-38 |
| HuLRRK2        | cd00180, S_TKc | 3.00E-30 |
|                | cd00192, PTKc  | 5.00E-36 |
| HuPKAalpha1    | cd00180, S_TKc | 3.00E-82 |
|                | cd00192, PTKc  | 3.00E-22 |
| SRC_Hu         | cd00192, PTKc  | 3.00E-104|
|                | cd00180, S_TKc | 9.00E-51 |

Myotubulin homologue sequences were identified, and protein kinase domains identified, as described in Methods. Protein kinase domains were subjected to analysis at NCBI CDD [40,41]. This table summarizes the returns for those searches - the query sequences, the hit models, and E values.

Models: cd00180, S_TKc (Serine/Threonine protein kinases, catalytic domain); cd00192, PTKc (Catalytic Domain of Protein Tyrosine Kinases).

0.90 in every case. Bootstrap support in Maximum Likelihood is weaker and more variable, depending more on details of alignment composition, but nevertheless the mean exceeds 80% for each group. Despite repeated attempts using distinct input alignments, data-transformation techniques (i.e. identifying and removing rapidly evolving sites [6]) and amino acid substitution models, we were unable to obtain consistent tree topologies with high support for deep interior branch points. This indicates a high degree of sequence divergence of the several myotubulin sub-types.

The domain architecture data presented in Figure 2 are for the most part consistent with the placement of the new myotubulin homologues into similarity clusters based on phylogenetic tree inference data. All of the new sequences placed into similarity groups have a full PH-GRAM domain, and a myotubulin phosphatase domain (predicted to be active or inactive) consistent with their class placement. The myotubulins of the “R5” group characteristically possess a DENN (Differentially Expressed in Neoplastic versus Normal cells) domain N-terminal to the PH-GRAM domain, and a PH (Plekstrin Homology) domain C-terminal to the phosphatase domain. This is true for the new sequences Tad51481 (Trichoplax) and Nv19357 (Nematostella). However sequence M001750622 (Monosiga) has an additional domain of unknown function at the extreme N-terminus, and lacks the C-terminal PH domain. This may indicate that the stable “R5” subunit domain architecture had not yet been achieved at this early stage of myotubulin gene evolution. Myotubulins of the “R3” group characteristically have a FYVE domain (Fab 1, YO1B, Vac 1, and EEA1 (early endosome antigen 1)) C-terminal to the phosphatase domain. This is true for sequence Tad64213 (Trichoplax). However, sequence N001631983 (Nematostella), also classified as an R3 member, lacks this domain. Furthermore, sequence N001626810 (Nematostella), classified as a member of the “R6” group, has a C-terminal FYVE domain. This is characteristically absent from the members of the R6 group, and for example, is absent from sequence Tad56124 (Trichoplax), also classified in this group. Thus it would appear that the Nematostella sequences in the R3 and R6 groups may have exchanged the FYVE domain. This may represent a novel, interesting genetic event in the evolution of the Nematostella myotubulin genes. Alternatively, it is conceivable that this might represent an error in genomic sequence assembly and annotation. Finally, the sequences M01745983C and Tad51955 are intriguing. These sequences cluster together consistently as a “new clade” in phylogenetic analysis based on alignments made from the PH-GRAM and phosphatase domains (see Legend to Table 2). In addition, each of them also possesses an N-terminal C2 domain (Protein kinase C Conserved region 2; phospholipid binding), which has not been reported previously in Metazoan myotubulins. This data supports the existence of a previously undescribed myotubulin architecture, perhaps restricted to Choanoflagellates and early Metazoa.

It is clear from the above phylogenetic analysis that even in the genome of Trichoplax, the most deeply diverging Metazoan known [19], there is a representative in each of the six typical myotubulin similarity groups. This pattern is continued throughout the rest of the Metazoans. This indicates that the gene diversification into the three catalytically active and three inactive myotubulin groups had been completed at the very base of the Metazoan clade.

The situation is less clear for the Monosiga genome. Representatives can only be identified clearly for the R5, R6, and R9 groups. This would suggest that the split between catalytically active and inactive myotubulins characteristic of the Metazoan clade had occurred.
already in the common ancestor of Choanoflagellates and Metazoans. However, it is impossible to propose a precise model for this process, as three similarity groups have no identified members. This might represent a genuine absence, and therefore have evolutionary significance. On the other hand, it is conceivable that the apparent absence of myotubularin gene types is an artefact of genome assembly and annotation. That this might be the case is supported by the discovery of the partial sequence Mbrevi5R3, which was manually constructed from unassembled genomic sequence reads. It therefore seems most prudent to say that the precise status of myotubularin genes in Choanoflagellates will have to await the completion of genome sequencing projects for other species in this group.

Accessory Protein Domains

Figure 6 presents a summary tabulation of the various domains found in myotubularin homologue sequences.
across the diverse eukaryotic groups examined in this study. It indicates the presence of active myotubularins with associated PH-GRAM domains in most species examined, across all the major supergroups. It summarises the occurrence of the inactive myotubularins without PH-GRAM domains in the Excavates, the IMLRK proteins in the Amoebozoans, and the inactive myotubularins with PH-GRAM domains in the Choanoflagellates and Metazoa. This figure also indicates that several types of accessory domains are also sometimes observed in myotubulin homologues.

Nearly all animal myotubularins characterized to date possess coiled-coil domains C-term to the myotubulin domain. These have been shown to be important in mediating the protein-protein interactions between myotubulin subunits [3], and might conceivably provide interaction sites for other protein partners. We find that the presence of coiled-coil domains is much more sporadic in the entire myotubulin set (see Figure 6 and also Figure 2), with many proteins lacking them. Where they occur, the most common location is C-term to the myotubulin domain, however a number of sequences, particularly in the Amoeboza, have N-term coiled-coil domains. The lack of coiled-coil domains in a number of myotubulin subunits suggests that coiled-coil domains might not always be needed for protein-protein interactions. These protein-protein interactions might also be mediated by the observed ANK [Ankyrin], LRR [Leucine-rich repeat], and WD40 domains.

Several domains are found which typically mediate membrane localization (PH, PX [Phox-like], C1, C2 [Protein kinase C conserved region 1 and 2], FYVE [Fab1, YO1B, Vac 1, and EEA1 (early endosome antigen 1)]), which is consistent with the postulated role of myotubulin proteins in vesicle transport. The presence of RhoGAP domains might indicate a role in direct regulation of the cytoskeleton.

A few sequences show a predicted transmembrane domain, and one has a predicted signal peptide. These are very unusual for myotubulin subunits, and would be consistent with localization in a particular intracellular membrane compartment, and entry into the endomembrane system, respectively.

Finally, several sequences contain a predicted nuclear localization signal (NLS). This was true for Nv109357 (Nematostella), and several other metazoan members of the R5 clade. This data is presented in Additional File 2. Amongst these sequences was the Drosophila homologue of MTMR5/MTMR13. This is consistent with the observation that this protein (originally called “Sbf1” [SET binding factor 1]) co-localizes with the epigenetic regulatory protein Trithorax (Trx) on polytene chromosomes [21]. The presence in several members of the R5 clade of a well-conserved basic sequence loop and NLS prediction together suggest that nuclear localization may be possible for other members of this group. In addition, we observed predicted NLS in two sequences from the plant Populus trichocarpa. This data is summarized in Additional File 3.

Several plants have been shown to contain a PH domain in the myotubulin subunits [3], and might conceivably provide interaction sites for other protein partners. We find that the presence of coiled-coil domains is much more sporadic in the entire myotubulin set (see Figure 6 and also Figure 2), with many proteins lacking them. Where they occur, the most common location is C-term to the myotubulin domain, however a number of sequences, particularly in the Amoeboza, have N-term coiled-coil domains. The lack of coiled-coil domains in a number of myotubulin subunits suggests that coiled-coil domains might not always be needed for protein-protein interactions. These protein-protein interactions might also be mediated by the observed ANK [Ankyrin], LRR [Leucine-rich repeat], and WD40 domains.

Several domains are found which typically mediate membrane localization (PH, PX [Phox-like], C1, C2 [Protein kinase C conserved region 1 and 2], FYVE [Fab1, YO1B, Vac 1, and EEA1 (early endosome antigen 1)]), which is consistent with the postulated role of myotubulin proteins in vesicle transport. The presence of RhoGAP domains might indicate a role in direct regulation of the cytoskeleton.

A few sequences show a predicted transmembrane domain, and one has a predicted signal peptide. These are very unusual for myotubulin subunits, and would be consistent with localization in a particular intracellular membrane compartment, and entry into the endomembrane system, respectively.

Finally, several sequences contain a predicted nuclear localization signal (NLS). This was true for Nv109357 (Nematostella), and several other metazoan members of the R5 clade. This data is presented in Additional File 2. Amongst these sequences was the Drosophila homologue of MTMR5/MTMR13. This is consistent with the observation that this protein (originally called “Sbf1” [SET binding factor 1]) co-localizes with the epigenetic regulatory protein Trithorax (Trx) on polytene chromosomes [21]. The presence in several members of the R5 clade of a well-conserved basic sequence loop and NLS prediction together suggest that nuclear localization may be possible for other members of this group. In addition, we observed predicted NLS in two sequences from the plant Populus trichocarpa. This data is summarized in Additional File 3.
the epigenetic regulatory Trithorax homologue protein ATX1 [22]. This has raised the question as to whether this protein might be able to enter the nucleus. Our finding of a conserved basic sequence region supports this possibility.

**Myotubularin Gene Expression in Entamoeba**

The unusually large complement of myotubularin homologues in *Entamoeba histolytica*, a well-known pathogenic organism, prompted us to explore the literature to examine patterns of myotubularin gene expression in this species. Davis et al. [23] reported differences in gene expression between the infective *E. histolytica* strain HM-1:IMSS and the non-pathogenic *E. histolytica* strain Rahman. Sequence EHI_141820 (one of the IMLRK proteins) showed an increase of 4.4× in expression (p = 2.4E-03), EHI_049780 [5.3×, p = 7.3E-03]. Genes for four inactive myotubularins also showed increases in expression (EHI_070120 [6.3×, p = 2.4E-03], EHI_140980 [6.3×, p = 5.0E-03], EHI_188050 [2.6×, p = 2.3E-03], EHI_185230 [10.7×, p = 7.4E-07], EHI_078170 [5.2×, p = 1.3E-04]). The latter two sequences are IMLRK proteins.

**Discussion**

A variety of experiments in animal and fungal systems including in vitro enzymatic studies, mutational analysis, complementation assays, and in vivo overexpression, agree in characterizing myotubularins as phosphatases of the D3 position in the inositol headgroup of inositol phospholipids. PI3P and PI(3,5)P2 appear to be primarily localized to the cellular endomembrane system and restricted domains of the plasma membrane, mediating transitions between endosomes and lysosomes, retrograde transport between the endosomal compartment and trans Golgi network, and endocytosis of some materials from the cell surface [1,3]. Mutations of animal and yeast myotubularins lead to abnormal accumulations of PI3P and PI(3,5)P2, apparently disrupting normal cellular membrane trafficking events, perhaps through abnormal concentrations and/or localizations of PI-phosphate specific membrane-binding effector proteins [2-4]. One would anticipate that such intracellular membrane trafficking processes, and the mechanisms regulating them, would be very ancient, having arisen quite early in eukaryotic evolution. This is consistent with our most common observation of a small number of myotubularin genes in organisms across a broad phylogenetic distribution, suggesting the presence of a single such gene in the last common ancestor for all extant eukaryotic groups. The PH-GRAM domain appears to be a very early acquisition, perhaps coincident with the divergence of a generic PTP domain into the characteristic elaborated myotubularin phosphatase domain.

Inactive myotubularin subunits are one of the particularly interesting features of this gene group. Our data are consistent with these having appeared on three separate occasions in eukaryotic evolution, in different taxonomic groups. The distinctive lack of a PH-GRAM domain in the inactive Excavate myotubularins makes it likely that these represent a unique lineage. Similarly, the IMLRK domain architecture of the Amoebozoa inactive myotubularins suggests they too have a unique origin. Finally, it is likely that an active myotubularin lineage then began an independent diversification event somewhere around the base of the Choanoflagellate/ Metazoan divergence to produce the six similarity groups characteristic of the Metazoans. This is consistent with our finding of all six myotubularin subgroups being identifiable in the deeply diverging Placozoa *Trichoplax*, but only three subgroup representatives being clearly identifiable from the Choanoflagellate *Monosiga*. More completed genome sequences from Choanoflagellates and even more deeply diverging protistan “animal allies” (e.g. Ichthyosporea and Filasterea [25,26]) will be necessary to precisely define this pivotal period in myotubularin gene history.

Myotubularin function has been most intensively studied in humans, where a number of diseases arising from inherited mutations have been characterized. It has been suggested that a common unifying pathophysiological mechanism in these disorders may be abnormality in the membrane trafficking necessary to alter the characteristic molecular composition and identity of the plasma membrane and specialized derivative membrane structures during cellular differentiation [4]. In this model the disordered membrane trafficking would be secondary to perturbations in the normal levels and perhaps subcellular distribution of PI3P and PI(3,5)P2, the normal substrates of myotubularins. This model suggests that the normal function of myotubularins becomes especially critical in situations where cells are required to turn over and alter, on a large scale, through membrane trafficking, the suites of proteins and perhaps lipids characterizing particular domains on the plasma membrane and components of the endomembrane system.

Myotubularin genes have undergone an expansion in the Amoebozoan species *Dictyostelium discoideum*. Nine myotubularins are predicted to be enzymatically active, and two inactive. Nothing is known about the function of the myotubularins in this organism. However, it is reasonable to suggest that they are involved in the regulation of
the substantial intracellular trafficking events that would accompany membrane reorganization during a complex life cycle. The two inactive *Dictyostelium* myotubularins also possess the distinctive IMLRK domain architecture. “ROCO” proteins (which usually contain LRR/ROCO/kine domains, but not myotubulin domains) were initially characterized in *Dictyostelium*, are biochemically best understood in this organism, but have a widespread phylogenetic distribution in both prokaryotes and eukaryotes [13]. In *Dictyostelium*, where there are 11 ROCO genes in all, functional evidence is available for four: gene gbpC is involved in chemotaxis; genes QkgA/roco2 and roco5 are involved in growth and development; and gene pats1 (our IMLRK sequence DDB0191503) is involved in cytokinesis. The ROCO proteins have recently received considerable attention because in humans the family member LRK2 is involved in familial and some cases of sporadic Parkinson’s disease [13]. Biochemical approaches, analysis of disease-associated mutations, and solved protein structures have revealed that the protein kinase domain is regulated by the GTPase activity of the ROC domain, through protein-protein dimerization mediated by the COR domain [15,27]. Thus these proteins have been likened to a “stand-alone” intramolecular signal transduction cascade, mediated by their multiple functional domains. *Dictyostelium* pats1 (DDB0191503) is essential for cytokinesis, and contains an enzymatically inactive myotubulin domain, whose function has not been experimentally tested. A reasonable proposal would be that the myotubulin-like portion of the protein could provide membrane localization via its PH-GRAM domain. It is known that specialized plasma membrane domains enriched in P(4,5)P2 accumulate at the intercellular bridge during cytokinesis, where they regulate the underlying actin cytoskeleton [28]. The *Dictyostelium* gene roco9 (DDB0191512) also encodes an IMLRK protein. Nothing is known about the function of this protein, but it contains a Rho-GAP domain, which might indicate a role in regulation of the actin cytoskeleton. Once again, the myotubularin-like region of the protein could supply membrane localization. Another functional possibility for the inactive myotubulin domains of both pats1 and roco9 is that they might bind to one or more of the many active *Dictyostelium* myotubulins, and mediate regulation of their activities. Several such combinations of active plus regulatory inactive myotubulin subunits are well characterized in animal cells [3].

In *Entamoeba*, another Amoebozoan, there is an even larger myotubulin gene set than observed in *Dictyostelium* - there are 8 active myotubulins, and 11 inactive myotubulins (9 of them with the IMLRK domain architecture). This is the largest collection of myotubulin genes observed to date in any eukaryotic genome examined. This large repertoire of active plus inactive subunits suggests the possibility of a particularly rich network of regulatory protein-protein associations. It is particularly striking that, in contrast to the intricate multicellular associations of *Dictyostelium*, the *Entamoeba* life cycle is morphologically rather simple. Underlying this apparently simplicity, however, is probably complex turnover and change to plasma membrane protein sets accompanying life cycle transitions and invasive contact with host tissues [29-31]. It might be hypothesized that the large complement of myotubulin genes found in this organism is necessary for precise spatial and temporal regulation of these membrane trafficking events, over and above the “constitutive” requirements of any eukaryotic cell. Their numbers would suggest that the IMLRK proteins might be particularly important. The data suggest that the protein kinase domains of the IMLRK proteins will be active, and that the ROC domains lack GTPase activity. This would indicate a change to the typical paradigm of ROC GTPase-mediated control of the kinase domain. It is possible that the divergent ROCO domains in these proteins effect protein kinase regulation via interaction with novel accessory proteins.

In most human cases of infection with *Entamoeba histolytica*, the organism remains in the lumen of the intestine, in contact with the epithelium. In a minority of cases, invasion of the intestinal wall occurs, which may lead to liver abscesses. The life cycle is completed by the organism forming cysts, which are released from the host in excrement, to infect new hosts. A significant increase in gene expression was noted in a myotubulin gene in a pathogenic vs a non-pathogenic strain of *E. histolytica* [23]. Significant upregulation was noted in several myotubulin genes which appear to be acting specifically in the encystment stage of the life cycle [24]. Taken together, these data suggest that myotubulin genes are important to both completion of the life cycle, and invasive disease in this organism.

**Conclusions**

We have presented a phylogenetic survey of myotubulin genes across a diverse array of eukaryotes, including distribution, domain architecture, and inferred evolutionary history. We have characterized an expansion of genes in the Amoebozoa encoding proteins with the novel combination of “IMLRK” (inactive myotubulin/ LRR/ROCO/kine) domains. This group is particularly prominent in the pathogenic organism *Entamoeba histolytica*, which contains the largest myotubulin gene family of any eukaryotic genome yet examined. Gene expression data in *E. histolytica* indicates that myotubulin function may be important to both critical life cycle transitions and host infection. The data indicate that pathogen myotubulin genes may be important targets for basic research, and perhaps novel strategies for disease control.
Methods

Identification of Putative Myotubularin Homologue Sequences

Sequences of all 14 human myotubularin proteins were obtained from NCBI Entrez [32]. A multiple sequence alignment was constructed and edited as presented in the next section. Eukaryotes with a completely sequenced genome were identified using the Genomes Online Database [33,34], and organismal protein data files were obtained from the sites linked therein. A Hidden Markov Model (HMM) of the human myotubularin multiple sequence alignment was constructed using the HMMER program package, which was then used to search the various eukaryotic protein sequence data files (program commands “hmmbuild”, “hmmpcalibrate” and “hmmssearch”, threshold E = 1). Candidate sequences were determined by a combination of low E value (generally less than E = 0.01) and a long alignment to the HMM model. A spreadsheet with the URLs of websites used to obtain protein datasets within which candidate myotubularin homologue sequences were found is presented in Additional File 4.

Determination of Myotubularin Similarity Regions within Sequences

Candidate myotubularin sequences obtained from the initial HMM search of protein data files were subjected to sequence:profile (FFAS03) [35,36] and profile:profile (HHPred) [37-39] analysis to identify the boundaries of the characteristic PH-GRAM and myotubularin phosphatase domains, by comparison with the solved structures of human MTMR2 (PDB: 1LW3, 1ZSQ). For most sequences this was a contiguous region, which was then utilized for multiple sequence alignment. FFAS03 returns standardized variable (“Z”) scores for comparisons between a query and a solved template structure sequence, with a score of 9.5 cited by the authors as being statistically significant. Candidate myotubularin sequences routinely exceeded this threshold. HHPred returns a probability score reflecting both the alignment between HMMs formed based on the query sequence and solved structure sequences, and predicted secondary structure. A probability of 95% is cited by the authors as having a very low false positive rate. Candidate myotubularin sequences routinely exceeded this threshold.

Characterization of Non-Myotubularin Domains within Sequences

Candidate myotubularin homologue sequences obtained by HMM search as described above were examined for functional domains using FFAS03 and HHPred as described above (except now using as a comparison all sequences with solved structures in the PDB), and also NCBI CDD [40,41], Pfam [42,43], and InterProScan [44,45], all with default settings. For the identification of ROCO domain sequences the comparison structure was that of the ROCO domain of Chlorobium tepidum (PDB: 3dpu_A [15]). The identity of the domains was confirmed by successive rounds of multiple sequence alignment (as detailed below), Hidden Markov Model construction (as detailed above), and database searching.

Characterization of Additional Protein Sequence Features

Candidate myotubularin homologue sequences obtained by HMM search as described above were examined for the presence of predicted signal peptides (Phobius [46,47], SignalP [48,49]), predicted transmembrane helices (Phobius [46,47], TMHMM [50,51]), predicted coiled-coil regions (Marcoil [52,53], PairCoil2 [54,55]), and nuclear localization signals NLStradamus [56,57]).

Multiple Sequence Alignment

Candidate Myotubularin Sequences

Candidate myotubularin sequences (including both the PH-GRAM domain and the myotubularin phosphatase domain, or just the phosphatase domain alone (as defined by the sequence of the solved structure of MTMR2_Hu (PDB:1LW3)) were aligned utilizing as necessary several multiple sequence alignment programs: Muscle [58], T-Coffee [59] or M-Coffee [60,61]. Quality of alignments was guided by evaluation at the T-Coffee web server. In some instances, sub-alignments were constructed, and then either sequences, or other sub-alignments were added using the Profile alignment mode of T-Coffee or ClustalX [62] (default program settings). Alignments were displayed and edited using the program GeneDoc [63]. After alignment analysis, it was found that some database sequences for candidate myotubularin homologues were incomplete due to annotation mistakes. These were supplemented with additional sequence by use of the appropriate organismal genome browser, and search of the organismal genomic DNA utilizing TBLASTN. Such sequences are denoted with the suffix “C” in the figure legends. For the reference multiple sequence alignment presented as Additional File 1 (100 sequences), no sequence regions were deleted.

Protein Kinase and ROCO Domains

Protein kinase domain and ROCO domain sequences within some myotubularin homologue candidates, detected as described above, were subjected to multiple sequence alignment with M-Coffee, displayed and edited with GeneDoc, as described above.

Phylogenetic Tree Inference

Multiple sequence alignments were constructed as detailed above. In some instances rapidly evolving sites (Category 8) were identified with PAML [64] and
removed from the alignment (analysis performed using the programs AIR-Identifier and AIR-Remover at the University of Oslo BioPortal http://www.bioportal.uio.no/).

Bayesian phylogenetic trees were inferred with Phylo-Bayes 3.2d [65]. Two independent Markov Chains were run under various amino acid substitution models, and between-sites rate variation models (UL3, Dirichlet; UL3, Uniform; WLSR5, Dirichlet) for approximately 5,000 cycles, using a 20% (approximately 1,000 cycle) burn-in. Chain convergence was checked using the statistics “maxdiff” < 0.10 and “effsize” > 100. Maximum likelihood trees were inferred with PhyML 3.0 [66] and PhyML-mixture [67]. A two-stage process was used [6], where first the best tree was inferred from 20 random starts, using SPR moves, from a Parsimony input tree (PhyML) or a BioNJ input tree (PhyML-mixture). Various amino acid substitution models and models for between site rate variation were used ([JTT plus 4 Gamma categories, empirical amino acid frequencies, proportion of invariant sites estimated], [WAG plus 4 Gamma categories, empirical amino acid frequencies, proportion of invariant sites estimated], [LG plus 4 Gamma categories, empirical amino acid frequencies, proportion of invariant sites estimated], [EX3, single rate category, model amino acid frequencies]). Then a second stage utilized the best tree from the first stage as a user input tree, and inferred 100 bootstrap replicates, using SPR moves, employing the same amino acid substitution and site rate variation parameters as in the first stage.

Additional material

Additional file 6: Additional Information on RCOO Sequence Alignment. This file presents species designations and database accession numbers for sequences presented in the multiple sequence alignment of Figure 4.

Abbreviations
ANK: Ankyrin domain; CDD: Conserved Domain Database; CMT: Charcot-Marie-Tooth; C1: Protein kinase C conserved region 1 (C1) domain (Cysteine-rich domain); C2: Protein kinase C conserved region 2 (C2) domain (Cysteine-rich domain); DENN: differentially expressed in neoplastic versus normal cells; FFAS: Fold and Function Assignment System; FIVYE: Fab 1, YO1B, Vac 1, and EEA1 (early endosome antigen 1); GAP: GTPase activating protein; GRAM: glucosyltransferases; Rab-like GTPase activators and tyrosinases; HHPred: HMM-HMM structure prediction; HMM: Hidden Markov Model; IMLRK: Inactive myotubularin/LRK/ROCO/Kinase domain architecture of Amoebobazoza, LRR: Leucine-rich repeat; MTMR: MT1M-related; NLS: Nuclear localization signal; PDB: Protein Data Bank; PH: Pleckstrin-homology; Pfam: Protein Families; PKA: Protein Kinase A; PTP: Protein tyrosine phosphatase; PX: Phox-like; Rho-GAP: Rho-GTPase Activating Protein; RCOO: Ras of complex proteins (ROC) + C-term of ROC (COR); TKL: Tyrosine kinase-like; WD40: structural motif of 40-43 amino acids in the beta subunit of G-proteins; XLMTM: X-linked myotubular myopathy

Acknowledgements
The authors thank Mhairi Nimick for assistance with figure composition. DK, GBGM and MN are supported by the Natural Sciences and Engineering Research Council of Canada, the Alberta Cancer Board, and the Alberta Ingenuity Carbohydrate Research Group.

Authors’ contributions
GBGM and DK conceived of the study. DK designed the implementation of the study. DK collected all sequence data, performed domain mapping, multiple sequence alignment, phylogenetic tree analysis, and mined published gene expression data. DK composed the data figures and tables. DK and GBGM wrote and approve the manuscript.

Received: 14 August 2009 Accepted: 24 June 2010
Published: 24 June 2010

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doi:10.1186/1471-2148-10-196

Cite this article as: Kerk and Moorhead: A phylogenetic survey of myotubulin genes of eukaryotes: distribution, protein structure, evolution, and gene expression. BMC Evolutionary Biology 2010 10:196.