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14. ABSTRACT: Merlin, the NF2 gene product, shares a substantial homology with the ezrin-radixin-moesin (ERM) proteins. The merlin and ERM proteins are thought to be key regulators of interactions between the actin cytoskeleton and the plasma membrane in Schwann cells and polarized cells. They act as important members of signal transduction pathways that control cell growth and participate in the sorting of membrane proteins during exocytic traffic. Unlike ERM. Merlin has a distinct function as a tumor suppressor; however, the mechanism by which marline functions as a tumor suppressor is poorly understood. *Drosophila melanogaster* provides a genetic and developmental system that is amenable to experiment manipulation and has been very valuable to the study of tumor genetics. The *Drosophila* homolog of merlin shares sequence and functional similarity to the human protein. We have shown that merlin plays an important role in the control of mitosis exit and in the determination of dorsal/ventral compartment border during wing imaginal disc development. Although merlin mutation did not seem to significantly affect the overall cell-cycle duration, merlin mutant displayed prolonged proliferation during the cell cycle. We showed that merlin is required for the determination of the wing morphology, and demonstrated a genetic interaction between merlin and porcupine, which controls the acetylation of the Wingless morphogen during the development of the wing imaginal disc. In addition, we showed a potential interaction between merlin and shibire, which is involved in wingless protein trafficking during early embryogenesis. Also, we found a role for merlin in spermatogenesis. Finally, we analyzed the origin and evolution of merlin, and identified a monophyletic origin of the merlin proteins with the root in early metazoa. Our results suggest a universal role of merlin in a wide range of metazoan.

15. SUBJECT TERMS:
Neurofibromatosis 2 NF2, NF2 Gene, merlin, ezrin-radixin-moesin (ERM), *Drosophila melanogaster*, mitosis exit, development imaginal disc, morphogen, protein trafficking, wingless, porcupine, shibire, spermatogenesis and evolution

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INTRODUCTION:

Neurofibromatosis type 2 (NF2) is a hereditary disorder characterized by the development of bilateral vestibular schwannomas and is caused by a defective tumor suppressor gene called the *neurofibromatosis type 2* (*NF2*) gene (Baser et al., 2003; Chang et al., 2005). The *NF2* gene encodes a protein named merlin for moesin-ezrin-radixin like protein. Merlin shares a great deal of homology with the ezrin, radixin, and moesin (ERM) proteins, which belong to the protein 4.1 superfamily of cytoskeleton-associated proteins that link cell surface glycoproteins to the actin cytoskeleton. Presently, the mechanism by which merlin functions as a tumor suppressor is poorly understood.

Drosophila melanogaster provides a genetic and developmental system, which is amenable to experimental manipulation, and has been very valuable to the study of tumor genetics. The *Drosophila* homolog of merlin shares sequence similarity to the human merlin protein (McCartney and Fehon, 1996; Fehon et al., 1997). In addition, the human *NF2* gene could rescue the lethal merlin mutant allele in *Drosophila*, implying a functional conservation (LaJeunesse et al., 1998). Molecular genetic analysis reveals that merlin is essential for regulation of proliferation and differentiation in the imaginal disc. However, understanding the tumor-suppressor function of merlin requires additional knowledge about specific cell-cycle points where merlin regulates proliferation and coordinates it with morphogenesis.

We have found that cells in the wing imaginal disc from the fly larva with a homozygous merlin mutation (mer^4) displayed abnormalities in the control of mitosis exit. Cytological images of mutant cells frequently showed asynchronous anaphase and telophase. We have also isolated adult mer^4 pharates. Interestingly, these *mer* mutant adults showed abnormal leg morphology. Some of them displayed a duplication of the wing disc, and in some cases, the dorsal/ventral compartment border in the *mer* wing disc was not detected. These results suggest that merlin is important not only for the control of mitosis exit but also for the determination/maintenance of global morphogenetic gradients in the wing imaginal disc.

The goal of our proposed research is to examine the novel role of merlin in the control of mitosis and development. Specifically, we plan to confirm the role of merlin in the control of mitosis and determine whether there are any additional points in the cell cycle where merlin executes its activity. We will examine the role of merlin in wing imaginal disc development and the effect of merlin mutation on specific regulatory protein expression within the wing imaginal disc. In addition, we will attempt to investigate whether the abnormalities in mitosis observed in merlin mutant fly can also be seen in mouse and human schwannoma cells lacking *NF2* function. From this study, we hope to a better understanding of how merlin executes regulation of proliferation and how it coordinates proliferation, mitosis, and morphogenesis. Future investigation of the signaling pathways that link merlin to intracellular signals regulating cell division may enable designs for novel therapeutic regiments to cure NF2 schwannomas and associated tumors.

BODY:

Aim 1: To conduct cytological analysis on additional merlin mutant alleles and allelic combinations for the control of mitosis exit and morphogenesis.

Task 1: In addition to mer^4, we have obtained merlin mutant alleles, including mer^1, mer^2, mer^3, and mer^4;mer+, from Dr. Rick Fehon at Duke University. These mutants have been maintained in the lab. Larvae with the hemizygous mer3 or mer4;mer+ genetic background were prepared. The wing imaginal discs and neural ganglia were isolated from these larvae for cytological analysis as previously described for the mer^4 mutant.

Task 2: We have initiated cytological analysis of tissues isolated from hemizygous mer3, mer4;mer+, and the parental strain containing the chromosome 2Pim in which the mer allele was induced. As shown in Table 1, *mer^4* mutant cells frequently displayed asymmetric anaphase-telophase figures with one cell in anaphase and the other in telophase, compared with the wild type strains Oregon and Lausenne. The asynchrony in anaphase-telophase transition appears not to depend on the balancer chromosome since the weak *mer^3* allele showed an intermediate degree of asymmetric figures. Importantly, the asynchronous phenotype in the anaphase-telophase transition frequently seen in the *mer^4* mutant was rescued by the addition of a *mer^+* chromosome. These results suggest that merlin is involved in the control of mitosis exit. However, when the parental 2Pim strain was analyzed, a high percentage of asymmetric anaphase-telophase figures was detected, excluding merlin mutation as the sole determinant for this mitotic abnormality. We are presently examining the cause of asymmetric anaphase-telophase figures in 2Pim.

Table 1. Mitotic asymmetry in different merlin alleles, compared with the parental 2Pim strain and two other wild-type controls Oregon and Lausenne.

Genetic Background	Number of anaphase and telophase cells analyzed	Number of cells with asymmetric anaphase-telophase figures (one cell in anaphase and the other in telophase.)	% of cells with asymmetric figures
mer^4	197	30-52	15.2-26.4
mer^4 **	157	20	12.7
mer^4; mer^+	181	1	0.5 *
mer^3	42	3-5	7.1-11.9
2Pim	86	12-28	14-32
Oregon	31	1	3.2
Lausenne	108	0	0

* An abnormal high frequency (47%) of the anaphase figures with symmetrically lagging chromatids was observed. In other genetic combinations studied, this parameter usually does not exceed 9.5%.

** mer^4 chromosome with another balancer.

To further analyze mitotic abnormalities, we have begun performing confocal microscopic examination. Whole imaginal discs were prepared from the *mer^4* hemizygous larvae and the wild-type HikkoneA/W strain, and stained with the antibody anti-H3p against phospho-histone 3, a marker for mitotic cells. Histone phosphorylation is mediated by the cyclinB/cdc2 protein complex whose kinase activity rises in prophase, reaches maximal in metaphase, and declines during anaphase-telophase. Thus the levels of the H3p protein reflect different stages of

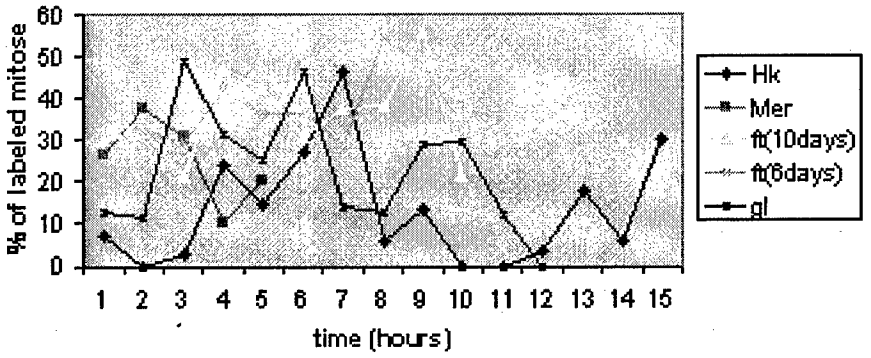
mitosis. By analyzing relative fluorescent signals of the H3p protein between sister nuclei, we detected *mer*^{4} cells that showed anaphase-telophase asymmetry. We plan to continue this confocal microscopy analysis employing other markers specific to each cell-cycle phase to examine the role of merlin in mitosis exit control.

Task 3: The task was proposed for the 3rd year study, and thus, has not been started.

Aim 2: To examine and compare the duration of the cell cycle and mitosis phases using various merlin mutants and to study subcellular localization of merlin at various phases of mitosis.

Task 4: We conducted a mitotic labeling experiment to measure the duration of the cell cycle and mitosis phases using imaginal discs of the wild-type Hikkone A/W strain, the strain with homozygous overgrowth mutation ft^4 (benign tumor), the strain with homozygous tumor-suppressor mutation l(2)gl^{DV275} (malignant tumor), and the strain with homozygous *mer*^{4} mutation (benign tumor). Figure 1 shows that the time between the two cell cycle peaks of wild-type cells is about 9h, consistent to those reported previously (Trunova et al., 1998, 2001; Dubatolova and Omelyanchuk, 2004).

Figure 1. The labeled mitosis curves for imaginal disc cells of the wild-type Hikkone A/W, l(2)gl^{DV275}, ft^4, and mer^4 strains. Two different ages (6 and 10 days) of ft^4 larvae were used.



The labeled mitosis curve for l(2)gl cells shows 3 peaks, instead of one peak observed in wild-type cells. The left peak constitutes the cell population with a shorter G2 phase than that in wild type. The middle peak represents the population with the same G2 duration as that in wild type. The right peak corresponds to the cell with a longer G2 phase. Similarly, two populations of ft^4 cells were detected, one with a shorter G2 phase and the other with a longer G2 phase. For mer^4 cells, a subpopulation of cells having a shorter G2 period was also seen. However, we could not cultivate *mer*^{4} imaginal discs for more than 5 h *in vitro*.

Task 5: To cultivate *mer*^{4} cells for a longer period of time, we prepared the Robb's complete tissue-culture medium (Ashburner, 1989). In our preliminary test, we successfully performed mitotic labeling by BrdU incorporation into chromatin of the wild type and *mer*^{4} imaginal disc cells, followed by anti-BrdU antibody staining. We are now in the process of determining the effect of merlin mutations on the duration of cell cycle phases.

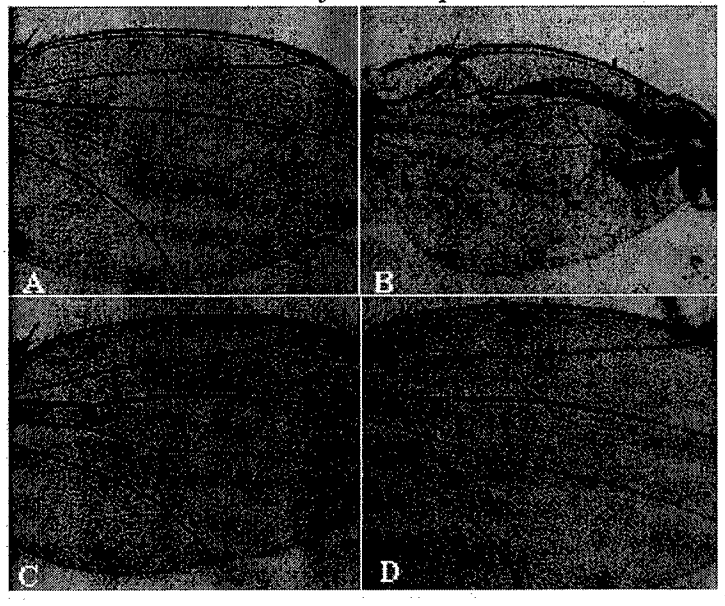
Task 6: To estimate the cell cycle duration in the wing imaginal disc, mosaic clone spots of multiple wing hairs (mwh) were induced in the +/mwh larvae at different developmental ages by 1000R of γ-rays for wild-type and mer^3 homozygotes. Irradiated larvae were grown to the adult stage. Adult male wings were removed and the spots were photographed and projected onto the map of adult wing. The clone dimension was determined by hair counting (Garcia-Bellido and Merriam, 1971; Gonzalez-Gaitan et al. 1994). By calculating clone frequency as a function of the time between egg laying and irradiation of larvae, we estimated the cell cycle duration to be 9.4h for wild type and 9.2h for mer^3. Thus, the overall cell-cycle duration appears to be not significantly affected by merlin mutation.

Task 7: This task was planed for years 2 and 3.

Aim 3: To further examine the role of merlin in the determination/maintenance of the D/V compartment border in the *Drosophila* wing imaginal disc and to investigate how merlin mutation affects the expression of proteins important for the determination of the compartment border.

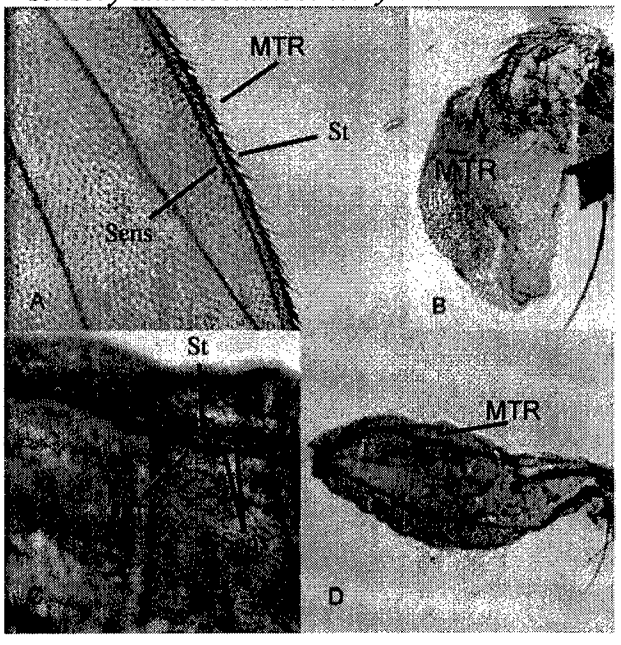
Task 8: We examined the effect of various merlin mutant alleles on the wing morphology. We examined pupal wings because the mer^4 allele does not yield adult flies. We found that the crossveins were completely absent (Figure 2B). Similarly, adult viable mer^3 individuals showed a complete loss of anterior and a partial loss of posterior crossveins (Figure 2A). With the use of the Gal4 driver 1096, which is active in the wing pouch region, ectopic expression of the UAS-mer+ transgene did not affect the wing morphology (Figure 2C). In contrast, over expression of UAS-mer-ΔBB, a Blue-Box deletion merlin construct, led to the reduction of both crossveins (Figure 2D). These results indicate that merlin plays a role in the determination of the wing morphology.

Figure 2. The wing morphology in different merlin alleles. (A) mer^3, (B) mer^4, (C) 1096;UAS-mer^+, and (D) 1096;UAS-mer-dBB. See the above text for description.



Task 9: To examine the role of merlin in the determination of the dorsal/ventral (D/V) compartment border in the wing imaginal disc, we first analyzed the effect of various merlin alleles on the expression and distribution of the Wingless (Wg) morphogen protein using the Gal4 driver 1096. Overexpression of the merlin protein with a deleted Blue Box (UAS-mer-ΔBB) did not alter the gross morphology of the wing (Figure 3A). In particular, the medial triple row (MTR) morphology, including the stout, sensory, and mechanosensory bristles were not affected. Interestingly, overexpression of procupine (porc), which controls the acetylation of the Wg protein (Tanaka et al., 2002), completely disrupted the wing morphology, displaying fragmented MTR structure (Figure 3B). Preliminary immunostaining experiments showed that porc overexpression resulted in the disappearance of Wg protein expression in the D/V compartment border (data not shown). Simultaneous overexpression of mer-ΔBB and porc restored the MTR structure (Figure 3C) and partially restored the overall wing morphology (Figure 3D). This phenotype was accompanied by the reappearance of Wg protein stripe in the D/V compartment border (not shown). These results suggest a potential interaction between merlin and porc during the development of the wing imaginal disc.

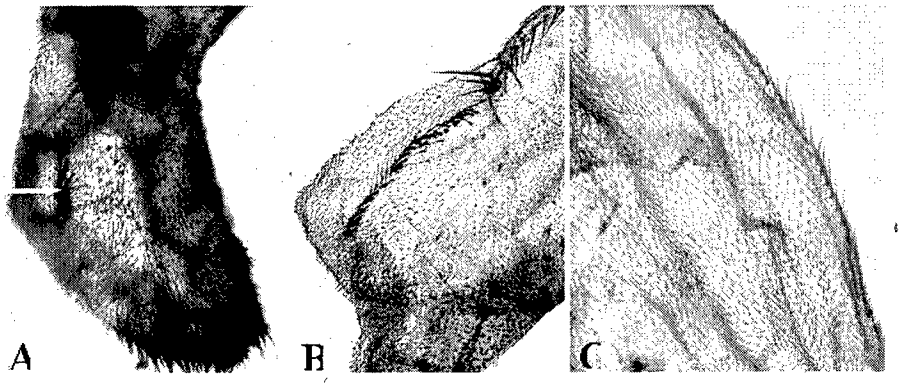
Figure 3. Merlin and porc genetically interact. (A) Overexpression of mer-ΔBB in the wing pouch using the Gal4 driver 1096 did not affect the general morphology of the wing with the exception of crossveins and the dimension of wing segments. In particular, the medial triple row (MTR) morphology including stout, sensory, and mechanosensory bristles were not affected. (B) Overexpression of porc disrupted the wing morphology; specifically the MTR structure became fragmented. (C) Overexpression of both porc and mer-ΔBB restored the MTR structure and partially recovered overall wing morphology (D). St - stout bristles; Sens - sensory and mechanosensory bristles.



Shibire (shi) is the dynamin protein participating in various microtubule processes (van der Bliek and Meyerowitz, 1991; Kitamoto, 2002). The shi protein participates in cytokinesis and endocytosis, and is known to be involved in Wg trafficking during early embryogenesis (Strigini and Cohen, 2000). We tested potential shi and

merlin interaction using similar Gal4-driven overexpression assays. As shown in Figure 4A, ectopic expression of a dominant-negative form of Shi, shi^{K44A} (Ramaswami et al., 1993), led to a completely disrupted wing morphology with no wing margin structure observed. Simultaneous overexpression of shi and mer-ΔBB restored the wing margin structure (Figure 4B). Moreover, overexpression of shi together with the mer^{+} gene, almost completely rescued the wing margin structure and restored the veins structure (Figure 4C). These results also suggest a potential interaction between merlin and shi during wing development.

Figure 4. Shibere and merlin genetic interaction. (A) 1096,UAS-shi^{K44A}. Arrow indicates occasional fragment of wing margin. (B)1096,UAS-shi^{R44F};1096,UAS-mer-dBB, C) 1096,UAS-shi^{R44F};1096,UAS-mer^{+}.



Previously, LaJeunesse et al. (1998) showed that the mer^{3} allele was viable but sterile. Since many proteins, which are involved in exocytosis/endocytosis, are also important for spermatogenesis, we examined the viable, but completely sterile, mutant mer^{3} for any defects in this process. Interestingly we found that the mer^{3} mutant showed abnormalities in male meiosis due to cytokinesis failure. During the cyst polarization (comet) stage, mer^{3} sperm nuclei displayed abnormal shape and failed to come together near the cyst wall (Omelyanchuk et al., 2005. Abstract presented to the 2005 CTF International Consortium for the Molecular Biology of NF1, NF2, and Schwannomatosis). Experiments are in progress to further examine how merlin participates in spermatogenesis.

Task 10: The task was proposed for years 2 and year 3, and has not been initiated.

Aim 4: To investigate whether *$NF2^{-/-}$* mouse schwannoma cells also show cytological abnormalities in mitosis similar to those seen in the *Drosophila* imaginal discs.

Task 11: We have made a collaborative agreement with Dr. Marco Giovannini of INSERM, France for his *$Nf2^{flox2/flox2}$* and P0Cre mice (Giovannini et al., 2000). These mice have been imported into our vivarium and bred to produce mice with conditional *Nf2*-deletion in Schwann cells.

Task 12: Presently, we are closely watching the P0Cre;*$Nf2^{flox2/flox2}$* mice develop schwannomas, which usually occur after 10 months of age.

Task 13: We are breeding a colony of *Nf2*$^{flox2/flox2}$ mice, which will be used to generate *Nf2*$^{flox2/+}$ heterozygous mice. Preparation of Schwann cell cultures will be performed once these mice are obtained in the next year.

Task 14: The experiment for cytological preparations of schwannoma cells and Schwann cells was proposed for years 2 and year 3, and has not been initiated.

Task 15: During the past year, three research abstracts were presented to national and local meetings. One research paper (Golovnina et al., 2005) will be published in *Evolutional Biology* (see attachment).

KEY RESEARCH ACCOMPLISHMENTS:

(1) We have confirmed mitosis exit abnormalities in merlin mutants. The abnormalities frequently seen in the *mer*^{4} mutant were rescued by the addition of a *mer*$^{+}$ chromosome the analysis. These results support our hypothesis that merlin plays a role in the control of mitosis exit.

(2) We estimated the cell cycle duration for wing imaginal disc cells of wild type and mer^{3} mutant. We found that the overall cell cycle duration was not significantly affected by merlin mutation. However, preliminary mosaic clone analysis revealed prolonged proliferation in mer^{3} cells during the cell cycle.

(3) Using overexpression assays, we showed that merlin is important for the determination of the wing morphology. We also demonstrated a genetic interaction between merlin and porcupine, which controls the acetylation of the Wingless morphogen during the development of the wing imaginal disc. In addition, we showed a potential interaction between merlin and shibire, a dynamin participating in cytokinesis and endocytosis and involving in Wingless protein trafficking during early embryogenesis.

(4) We demonstrated for the first time that merlin is important in spermatogenesis. The viable, but sterile mer^{3} mutant displays abnormalities in male meiosis due to cytokinesis failure.

(5) By combining bioinformatics and phylogenetic approaches, we demonstrated a monophyletic origin of the merlin proteins with the root in early metazoa. We identified conservation of several functionally important sites among all merlin proteins. Our data suggest a universal role of merlin in a wide range of metazoa.

REPORTABLE OUTCOMES:

Three research abstracts were presented to national and local

meetings during the past year. Also, one research paper will be published in the journal *Evolutionary Biology*.

Abstracts

(1) Omelyanchuk, L.V., Dorogova, N.V., Kopyl, S., Akhmameteva, E.M., Perceva, J., Fehon, R.G., and Chang, L.S. 2005. The Role of Merlin in *Drosophila* Spermatogenesis. Abstract presented to the 2005 CTF International Consortium for the Molecular Biology of NF1, NF2, and Schwannomatosis.

We reported that merlin plays important role in spermatogenesis. By examining the viable, but completely sterile, merlin mutant mer^3 for any defects in this process, we found that the mer^3 mutant showed abnormalities in male meiosis due to cytokinesis failure. During the cyst polarization (comet) stage, mer^3 sperm nuclei displayed abnormal shape and failed to group near the cyst wall. Preliminary immunolocalization experiments suggested that merlin might be involved in the control of acrosome-nucleus association and/or participate in the process of nucleus migration and condensation during cyst polarization.

(2) Golovnina, K., Blinov, A., Akhmametyeva, E.M., Omelyanchuk, L.V., and Chang, L.-S. 2005. Evolution and Origin of Merlin, the Product of the *Neurofibromatosis Type 2* Tumor-Suppressor Gene. Abstract presented to the 2005 CTF International Consortium for the Molecular Biology of NF1, NF2, and Schwannomatosis.

By combining bioinformatics and phylogenetic approaches, we demonstrated a monophyletic origin of the merlin proteins with the root in early metazoa. Conservation of several functionally important sites among all merlin proteins suggests a universal role of merlin in a wide range of metazoa.

(3) Akhmametyeva, E.M., K. Golovnina, A. Blinov, L.V. Omelyanchuk, and L.-S. Chang. 2005. Evolution and Origin of Merlin, the Product of the *Neurofibromatosis Type 2* Tumor-Suppressor Gene. The 7th Annual Comprehensive Cancer Center Scientific Meeting, Columbus, OH

We presented this research abstract to our University Cancer Center annual meeting. The finding is the same as the abstract # 1 presented to the 2005 CTF International Consortium for the Molecular Biology of NF1, NF2, and Schwannomatosis.

Publication and Manuscript

(1) Golovnina, K., Blinov, A., Akhmametyeva, E.M., Omelyanchuk, L.V., and Chang, L.-S. 2005. Evolution and Origin of Merlin, the Product of the Neurofibromatosis Type 2 Tumor-Suppressor Gene. *Evolutionary Biology*, In Press.

In this article, we combined bioinformatics and phylogenetic approaches to demonstrate that merlin homologs are present across a wide range of metazoan lineages. While the phylogenetic tree shows a monophyletic origin of the ERM family, the origin of the merlin proteins is robustly separated from that of the ERM proteins. The derivation of merlin is supposed in early metazoa. We have also observed the expansion of the ERM-like ancestors within the vertebrate clade that occurred after its separation

from Urochordata (Ciona intestinalis). Amino acid sequence alignment reveals the absence of an actin-binding site at the C-terminal domain of all merlin proteins compared with the rest of the ERM members. However, a more conserved pattern of amino acid residues is found in the so-called "Blue Box" region, although some amino acid substitutions are located in the merlin sequences from worm, fish, and Ciona. Examination of sequence variability at functionally significant sites, including the serine-518 residue, the phosphorylation of which modulates merlin's intra-molecular association and function as a tumor suppressor, identifies several potentially important sites that are conserved among all merlin proteins but divergent in the ERM proteins. Furthermore, analysis of the evolution of the merlin gene structure reveals the existence of common NF2 splicing variants in human and Caenorhabditis elegans. In summary, our results demonstrate a monophyletic origin of the merlin proteins with the root in early metazoa. Conservation of several functionally important sites among all merlin proteins suggests a universal role of merlin in a wide range of metazoa.

CONCLUSIONS:

To better understand merlin functions in mitosis and development, we studied *Drosophila melanogaster*, which provides a genetic and developmental system that is amenable to experimental manipulation and has been very valuable to study tumor genetics. We have shown that merlin plays an important role in the control of mitosis exit and in the determination of dorsal/ventral compartment border during wing imaginal disc development. Although the overall cell cycle duration appears not to be significantly affected by merlin mutation, the merlin mutant displays prolonged proliferation during the cell cycle. We also show that merlin is important for the determination of the wing morphology, and demonstrate a genetic interaction between merlin and porcupine, and between merlin and shibire. We have also found a role for merlin in spermatogenesis. Finally, we have analyzed the origin and evolution of merlin, and identified *a monophyletic origin of the merlin proteins with the root in early metazoa. Furthermore, our results suggest a universal role of merlin in a wide range of metazoa.*

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ABSTRACT

Merlin, the *NF2* gene product, shares a substantial homology with the ezrin-radixin-moesin (ERM) proteins. The merlin and ERM proteins are thought to be key regulators of interactions between the actin cytoskeleton and the plasma membrane in Schwann cells and polarized cells. They act as important members of signal transduction pathways that control cell growth and participate in the sorting of membrane proteins during exocytic traffic. Unlike ERM, merlin has a distinct function as a tumor suppressor; however, the mechanism by which merlin functions as a tumor suppressor is poorly understood. *Drosophila melanogaster* provides a genetic and developmental system that is amenable to experimental manipulation and has been very valuable to the study of tumor genetics. The *Drosophila* homolog of merlin shares sequence and functional similarity to the human merlin protein. We have shown that merlin plays an important role in the control of mitosis exit and in the determination of dorsal/ventral compartment border during wing imaginal disc development. Although merlin mutation did not seem to significantly affect the overall cell-cycle duration, the merlin mutant displayed prolonged proliferation during the cell cycle. We also showed that merlin is required for the determination of the wing morphology, and demonstrated a genetic interaction between merlin and porcupine, which controls the acetylation of the Wingless morphogen during the development of the wing imaginal disc. In addition, we showed a potential interaction between merlin and shibire, a dynamin participating in cytokinesis and endocytosis, and involving in Wingless protein trafficking during early embryogenesis. Also, we found a role for merlin in spermatogenesis. Finally, we analyzed the origin and evolution of merlin, and identified a monophyletic origin of the merlin proteins with the root in early metazoa. Our results suggest a universal role of merlin in a wide range of metazoa.

APPENDICES:

Three Abstracts and One Publication

Abstract presented to the 2005 CTF International Consortium for the Molecular Biology of NF1, NF2, and Schwannomatosis

• **Translating Basic Science to Clinical Treatments of Childhood**	• The tumor micro-environment	• **Signaling and Cytoarchitecture**
• **Translating Pathways to Therapies**	• **Bench to bedside: finding cures today**	• **OTHER**

ABSTRACT FORM

TITLE: The Role of Merlin in *Drosophila* Spermatogenesis

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The Role of Merlin in *Drosophila* Spermatogenesis. Leonid V. Omelyanchuk,[1] Natalia V. Dorogova,[1] Sergey Kopyl,[1] Elena M. Akhmameteva,[2] Julia Perceva,[1] Rich G. Fehon,[3] and Long-Sheng Chang.[2] *[1]Institute of Cytology and Genetics, Russian Academy of Sciences, Novosibirsk, Russia, [2]Department of Pediatrics, Children's Hospital and The Ohio State University, Columbus, Ohio, and [3]Department of Molecular Genetics & Cell Biology, University of Chicago, Chicago, Illinois, USA*

The *Drosophila* homolog of merlin, the human *neurofibromatosis-2* (*NF2*) gene product, shares a great deal of homology with the ezrin, radixin, and moesin (ERM) proteins. The ERM and merlin proteins are thought to be key regulators of interactions between the cytoskeleton and the plasma membrane in polarized cells. They act as important members of signal transduction pathways that control cell growth and participate in the sorting of membrane proteins during exocytic traffic. Since many proteins involved in exocytosis/endocytosis are important for spermatogenesis, we examine the viable, but completely sterile mutant mer^3 ($Met^{177}\rightarrow Ile$) for any defects in this process. Males hemizygous for mer^3 have seminal vesicles but are almost free of sperms. Squashed acetoorcein preparation of testes showed that the sperm cyst from the mer^3 male contained fewer sperm heads than that from the wild-type male. Although most mer^3 cells underwent normal meiotic divisions, some displayed two unequal-sized nuclei with with nebenkern (the mitochondrial body) reflecting chromosome nondisjuction, two equal-sized nuclei but with two nebenkerns in one cell displaying cytokinesis failure, tripolar spindles indicating non-coordinated nuclear and centrosome cycles, or 4-polar spindles. These morphological abnormalities bear some similarities to those seen in the ff16 meiotic mutant, suggesting incomplete cytokinesis in meiosis. The study of sperm individualization was performed using DAPI to stain the nucleus and antibodies specific for F-actin to visualize the actin cone bundles, referred as the individualization complex (IC) or membrane cytoskeletal complex, normally associated with the cystic bulge, a structure where sperm tails leave common cytoplasm. Interestingly, both the nuclei and actin cone bundles were abnormally distributed in the mer^3 cyst during sperm individualization. Not all nuclei within a mer^3 cyst had normal needle-like shape of sperm heads; instead, many of them have round-shaped sperm head. Normally the actin cone bundles are formed near the sperm heads and then moves toward the other end. Two groups of genes in *Drosophila* have been identified that affect this process. One is involved in IC formation and the other affects IC translocation. Neither IC formation nor IC translocation were found to be affected by the mer^3 mutation. These results suggest that merlin is important only for the distribution of sperm nuclei at the early stage of spermatogenesis. To examine any abnormalities at the cyst polarization stage, also called the comet stage, we used the expression of histone H2-GFP fusion protein to mark the nuclei and examined the living and DAPI-stained cysts. We found that in contrast to the wild-type cyst, the mer^3 cyst displayed abnormal cyst polarization; some nuclei failed to group at a pole and condensation of sperm nuclei frequently did not occur. Immunostaining using anti-merlin antibodies revealed that the merlin protein was localized near the cytoplasmic membrane region of cells in the apical zone of the wild-type cyst. In contrast, merlin was distributed throughout the cytoplasm in granule forms in spermatocytes of the mer^3 testis. During meiotic divisions, merlin first appeared as an organized compact body near the nuclei, and at the onion stage or later, it was re-distributed uniformly through the cyst. Also, merlin was normally detected in acrosomes situated at the end of the sperm head. However, merlin signal in the acrosome was found dissociated from the sperm head in the mer^3 cyst. After sperm individualization, merlin was detected in the so-called "waste bag," a structure where the extruded syncytial cytoplasm and other debris are removed after sperm individualization. These results suggest that merlin may be involved in the control of acrosome-nucleus association and/or participate in the process of nucleus migration and condensation during cyst polarization. (Supported by the US Department of Defense Neurofibromatosis Research Program)

Abstract presented to the 2005 CTF International Consortium for the Molecular Biology of NF1, NF2, and Schwannomatosis

• Translating Basic Science to Clinical Treatments of Childhood	• The tumor micro-environment	• Signaling and Cytoarchitecture
• Translating Pathways to Therapies	• Bench to bedside: finding cures today	• OTHER

ABSTRACT FORM

TITLE: Evolution and Origin of Merlin, the Product of the *Neurofibromatosis Type 2* Tumor-Suppressor Gene

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Evolution and Origin of Merlin, the Product of the *Neurofibromatosis Type 2* (*NF2*) Tumor-Suppressor Gene. Kseniya Golovnina[1], Alexander Blinov[1], Elena M. Akhmametyeva[2], Leonid V. Omelyanchuk[1], and Long-Sheng Chang[2], *[1]Institute of Cytology and Genetics, Russian Academy of Sciences, Novosibirsk, Russia, and [2]Center for Childhood Cancer, Children's Research Institute, Children's Hospital and Department of Pediatrics, The Ohio State University, Columbus, Ohio, USA*

Background: Merlin, the product of the neurofibromatosis type 2 (*NF2*) tumor suppressor gene, belongs to the ezrin–radixin–moesin (ERM) subgroup of the protein 4.1 superfamily, which links cell surface glycoproteins to the actin cytoskeleton. While merlin's functional activity has been examined in mammalian and *Drosophila* models, there is little understanding of its evolution, diversity, and overall distribution among different taxons.

Results: By combining bioinformatics and phylogenetic approaches, we demonstrate that merlin homologs are present across a wide range of metazoan lineages. While the phylogenetic tree shows a monophyletic origin of the ERM family, the origin of the merlin proteins is robustly separated from that of the ERM proteins. The derivation of merlin is supposed in early Metazoa. We have also observed the expansion of the ERM-like ancestors within the vertebrate clade that occurred after its separation from Urochordata (*Ciona intestinalis*). Amino-acid sequence alignment reveals the absence of an actin-binding site at the C-terminal domain of all merlin proteins compared with the rest of the ERM members. However, a more conserved pattern of amino acid residues is found in the so-called "Blue Box" region, although some amino-acid substitutions are located in the merlin sequences from worm, fish, and Ciona. Examination of sequence variability at functionally significant sites including the serine-518 residue, phosphorylation of which modulates merlin's intra-molecular association and function as a tumor suppressor, identifies several potentially important sites that are conserved among all merlin proteins but divergent in the ERM proteins. Furthermore, analysis of the evolution of the merlin gene structure reveals the existence of common *NF2* splicing variants in human and *Caenorhabditis elegans*.

Conclusion: These results demonstrate a monophyletic origin of the merlin proteins with the root in early metazoa. Conservation of several functionally important sites among all merlin proteins suggests a universal role of merlin in a wide range of metazoa.

- Supported by the US Department of Defense Neurofibromatosis Research Program.

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Evolution and Origin of Merlin, the Product of the *Neurofibromatosis Type 2* (*NF2*) Tumor-Suppressor Gene. Elena M. Akhmametyeva,[1] Kseniya Golovnina,[2] Alexander Blinov,[2] Leonid V. Omelyanchuk,[2] and Long-Sheng Chang[1] [1](Molecular Biology Program) Center for Childhood Cancer, Children's Research Institute and Department of Pediatrics, The Ohio State University, Columbus, OH 43205, USA, and [2]Institute of Cytology and Genetics, Russian Academy of Sciences, 630090, Novosibirsk, Russia

Merlin, the product of the neurofibromatosis type 2 (*NF2*) tumor suppressor gene, belongs to the ezrin–radixin–moesin (ERM) subgroup of the protein 4.1 superfamily, which links cell surface glycoproteins to the actin cytoskeleton. While merlin's functional activity has been examined in mammalian and *Drosophila* models, there is little understanding of its evolution, diversity, and overall distribution among different taxons. By combining bioinformatics and phylogenetic approaches, we demonstrate that merlin homologs are present across a wide range of metazoan lineages. While the phylogenetic tree shows monophyletic origin of the ERM family, the origin of the merlin and merlin-like proteins is robustly separated from that of the ERM and ERM-like proteins. The derivation of merlin is supposed in early Metazoa. We have also observed the expansion of the ERM-like ancestors within the vertebrate clade that occurred after its separation from Urochordata (*Ciona intestinalis*). Amino-acid sequence alignment reveals the absence of an actin-binding site at the C-terminal domain of all merlin or merlin-like proteins compared with the rest of the ERM members. However, more conserved pattern of amino acid residues is found at the so-called "Blue Box" region, although some amino-acid substitutions are found in the merlin sequences from worm, fish, and Ciona. Examination of sequence variability at functionally significant sites including the serine residue at position 518, phosphorylation of which modulates merlin's intra-molecular association and function as a tumor suppressor, demonstrates several potentially important sites that are conserved among all merlins but divergent in the ERM proteins. Taken together, these results suggest a universal role of merlin in a wide range of metazoan.

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Evolution and Origin of Merlin, the Product of the *Neurofibromatosis Type 2* (*NF2*) Tumor-Suppressor Gene

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Running title: Evolution and Origin of Merlin

Keywords: Merlin, the neurofibromatosis type 2 (NF2) gene, ezrin-radixin-moesin (ERM), phylogeny, BLAST

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ABSTRACT

Background: Merlin, the product of the *neurofibromatosis type 2* (*NF2*) tumor suppressor gene, belongs to the ezrin–radixin–moesin (ERM) subgroup of the protein 4.1 superfamily, which links cell surface glycoproteins to the actin cytoskeleton. While merlin's functional activity has been examined in mammalian and *Drosophila* models, there is little understanding of its evolution, diversity, and overall distribution among different taxons.

Results: By combining bioinformatics and phylogenetic approaches, we demonstrate that merlin homologs are present across a wide range of metazoan lineages. While the phylogenetic tree shows a monophyletic origin of the ERM family, the origin of the merlin proteins is robustly separated from that of the ERM proteins. The derivation of merlin is supposed in early Metazoa. We have also observed the expansion of the ERM-like ancestors within the vertebrate clade that occurred after its separation from Urochordata (*Ciona intestinalis*). Amino-acid sequence alignment reveals the absence of an actin-binding site at the C-terminal domain of all merlin proteins compared with the rest of the ERM members. However, a more conserved pattern of amino acid residues is found in the so-called "Blue Box" region, although some amino-acid substitutions are located in the merlin sequences from worm, fish, and Ciona. Examination of sequence variability at functionally significant sites including the serine-518 residue, phosphorylation of which modulates merlin's intra-molecular association and function as a tumor suppressor, identifies several potentially important sites that are conserved among all merlin proteins but divergent in the ERM proteins. Furthermore, analysis of the evolution of the merlin gene structure reveals the existence of common *NF2* splicing variants in human and *Caenorhabditis elegans*.

Conclusion: These results demonstrate a monophyletic origin of the merlin proteins with the root in early metazoa. Conservation of several functionally important sites among all merlin proteins suggests a universal role of merlin in a wide range of metazoa.

Background

The advancement in genome sequencing projects, accumulating knowledge in bioinformatics together with molecular genetic analysis of genes and their functions in a variety of model organisms, provides us an unprecedented opportunity to identify novel genes based on sequence relatedness to characterized genes [1]. This process is conducted using pair-wise sequence comparison with the understanding that genes form families wherein related sequences likely have similar functions. Although initial identification of the new genes may not yield a clear indication of their respective functions, studies on their evolution may allow validating their sequence identity and providing information on their putative functional characteristics. For genes evolved from duplication and/or adapted to different evolutionary niches during speciation, detailed sequence comparison can provide additional information on their biological and biochemical characteristics [2].

Neurofibromatosis type 2 (NF2) is a highly penetrant, autosomal dominant disorder with the hallmark being the development of bilateral vestibular schwannomas [3.4]. The tumor suppressor gene associated with NF2 has been identified and termed the *neurofibromatosis type 2* gene (*NF2*) [5,6]. The *NF2* gene encodes a protein named merlin for moesin-ezrin-radixin like protein, or schwannomin, a word derived from schwannoma, the most prevalent tumor seen in NF2. For simplicity, we refer to the *NF2* gene product as merlin hereafter.

Merlin shares a great deal of homology with the ezrin, radixin, and moesin (ERM) proteins, which belong to the protein 4.1 superfamily of cytoskeleton-associated proteins that link cell surface glycoproteins to the actin cytoskeleton [7,8]. Like the ERM proteins, merlin consists of three predicted structural domains [5,6,9]. The N-terminal domain, termed FERM (F for 4.1) domain, is highly conserved among all members of the ERM family and important for interactions with cell surface glycoproteins, including CD44 and intercellular adhesion molecules [10-13]. The second half of the molecule contains a predicted α-helical domain, which is also present in the ERM proteins. The unique C-terminus of merlin lacks the conventional actin-binding domain found in the ERM proteins. However, merlin can directly binds actin using the residues at the N-terminal domain and indirectly through its association with βII-spectrin or fodrin [14-16].

The merlin and ERM proteins are thought to be key regulators of interactions between the actin cytoskeleton and the plasma membrane in polarized cells. They act as important members of signal transduction pathways that control cell growth and participate in the sorting of membrane proteins during exocytic traffic [17,18]. However, unlike the ERM proteins, merlin has a distinct function as a tumor suppressor [19]. Growth suppression by merlin is dependent on its ability to form intramolecular associations [20,21]. In this regard, merlin exists in an 'open' (inactive form) or 'closed' (active growth-suppressive form) conformation that is regulated by phosphorylation [22-27].

While studies have been focused mostly on the functional analysis of merlin, limited information is available about its overall distribution across eukaryotes and about its evolution. A phylogenetic study indicates that the FERM domains of ERM homologs from sea urchin, *Caenorhabditis elegans*, *Drosophila melanogaster* and vertebrates share 74-82% amino-acid identity and have about 60% identity with those of merlin [17,28-34]. These levels of identity are exceptionally high, implying that the protein structure of the merlin and ERM proteins from these species may be well conserved. The most divergent ERM proteins are found in tapeworms and schistosomes. The FERM domains of these parasite proteins share only 44-58% similarity to their vertebrate homologs. The high degree of structural conservation among these proteins points to possible similarities or redundancy in functions. Intriguingly, no FERM domain-encoding genes have been identified in the genome of the baker yeast *Saccharomyces cerevisiae*, implying that FERM domains evolved in response to multicellularity, rather than as a cytoskeletal component [17].

In the present study, we have undertaken to expand our understanding of the taxonomic diversity of merlin and their phylogenetic relationships using experimentally annotated and predicted sequences. By the integration of the BLAST-based analysis using the available partial and whole genome sequences with phylogeny reconstruction, we have constructed an evolutionary tree for the entire ERM-family members from various taxons, and identified some interesting facts about their phylogenetic origin. In addition, we have also compared sequence variability at functionally significant sites including the phosphorylation site of merlin, and examined the exon-intron structural evolution of the *NF2* gene.

Results and Discussion

BLAST identification of merlin sequences. To identify putative merlin and ERM sequences in a wide range of eukaryotes, we performed BLAST analysis of 15 available genome databases. By searching through all annotated proteins and genome sequences, we identified 50 sequences from 30 species. Table 1 summarizes the full list of the predicted and annotated merlin and ERM proteins identified, and their GenBank accession numbers and related resources. No merlin-like sequences were found in the genomes of fungi and plants, as well as in those of Protozoa. On the other hand, while the sequencing projects of the hard ticks are still ongoing at The Institute for Genomic Research (TIGR), amino-acid sequences deduced from partial cDNAs of salivary glands that share a similarity to the FERM domain of merlin have been noted from *Rhipicephalus appendiculatus* (http://www.tigr.org/tigr-scripts/tgi/est_report.pl?GB=CD797075&species=r_appendiculatus), *Amblyomma variegatum* (http://www.tigr.org/tigr-scripts/tgi/est_report.pl?GB=BM291669&species=a_variegatum), and *Boophilus microplus* (http://www.tigr.org/tigr-scripts/tgi/est_report.pl?GB=CK190110&species=B.microplus).

Assembly of predicted merlin sequences from whole genomes shotgun. To date, the genomes of caenorhabditis remanei and drosophila yakuba are represented by a set of contigs (http://genome.wustl.edu/blast/client.pl). When contigs are ordered, oriented and positioned with respect to each other by mate-pair reads, they are known as a scaffold. Scaffolds are the main product of the whole genome shotgun strategy and can be assigned to chromosomes using chromosome specific markers. Although the extensive scaffolds for the genomes of caenorhabditis remanei and drosophila yakuba currently are not available, we were able to assembly predictive protein sequences most resembling to the merlin sequence of the closely related organism, caenorhabditis elegans or drosophila melanogaster, respectively, using tblastn search across the available set of contigs. We identified within the drosophila yakuba contig 49.37 a predicted merlin sequence, which is nearly identical to that of the drosophila melanogaster protein with the exception of three positions at the c-terminus, two substitutions at glu^{468}→asp and asn^{579}→ser, and an insertion of lys at position 575. Also, we found three

caenorhabditis remanei contigs, 564.6, 2151.1, and 2151.2, which contained merlin-like sequences with similarity ranging from 81% to 100% to the caenorhabditis elegans counterpart. It should be noted that the deduced amino acid sequences were assembled manually and in some cases, only partial or approximate amino acid sequences could be obtained. Nevertheless, they were useful for the identification of the definite gene in the respective genome and for the following phylogenetic reconstruction to validate their functional relationship and evolution.

Construction of a phylogenetic tree for the ERM family of proteins. To understand the origin and evolution of merlin, we conducted a phylogenetic analysis on the 50 proteins of the ERM family identified from 30 different taxa (Table 1) using the neighbor-joining method [35,36] combined with the molecular evolutionary genetics analysis program MEGA2 [37]. Three protein 4.1 sequences from human, mouse and zebra fish, respectively, were used as an outgroup. By comparing the bootstrap support value, which denotes the number of times a grouping occurring out of 1,000 random samples from the alignment, we constructed a phylogenetic tree for the ERM family of proteins (Figure 1). Based on the phylogenetic analysis, the entire ERM family can be subdivided into the ERM clade and the merlin clade. While both clades show a strongly supported monophyletic origin, the merlin clade can be robustly delineated and separated from the ERM clade (bootstrap support value = 100). Altogether, we identified 22 sequences for the merlin clade and 28 sequences for the ERM clade. The topology of the phylogenetic tree within the merlin clade appears to be in agreement with a general concept of the evolution history.

The merlin clade can be further divided into three groups according to the order of derivation: worms, insects, and Chordata with the earliest separated genus Ciona in the last taxonomic unit. The predicted merlin-like sequence from *Caenorhabditis remanei* is branched with that of *Caenorhabditis elegans,* and so is that of *Drosophila yakuba* from the *Drosophila melanogaster* counterpart. Both the so-called "unnamed protein 1" of *Tetraodon nigroviridis* and the so-called "unknown protein" of *Xenopus laevis* from the GenBank database are clustered into the Chordata merlin-like group with high bootstrap probabilities (Figure 1), confirming their identity as merlin homologs. The protein fragment from *Anopheles gambiae* bearing a sequence similarity to merlin is grouped together with the *Apis*

mellifera merlin-like protein with a bootstrap support value of 100.

Although the ERM-like proteins have been identified in *Taenia saginata*, *Schistosoma japonicum*, *Echinococcus granulosus*, and *Echinococcus multilocularis* [28-31], we did not found any merlin-like sequence in the genomes of these species. The lack of the merlin-like sequence in these parasite genomes may be due to incomplete genome sequences in the database. However, this is not likely because the merlin-like sequence was also not found in the genome of *Schistosoma mansoni*, which has been extensively studied. Another possibility is that the lost of merlin-like sequences in these organisms may reflect their response to parasitic lifestyle and reduction of various organ systems. Alternatively, the merlin protein may emerge later during evolution. In addition, no merlin-like sequence was found in the complete genomes of protozoans, fungi, and plants. Based on these results, we suppose that the derivation of merlin occurred in early Metazoa after its separation from flatworms.

As illustrated in the ERM clade (Figure 1), the ERM-like proteins found in the parasites can be grouped together but form a separate branch from the rest of ERM proteins. The clustering of the so-called "unnamed protein 2" of *Tetraodon nigroviridis* with the *Fugu rubripes* radixin protein defines its characteristics as a radixin-like protein (Figure 1). It should be noted that the two predicted ERM proteins, erm1a and erm1b, of *Caenorhabditis elegans* (http://www.wormbase.org/db/gene/gene?name=F42A10.2a;class=Transcript) may represent different isoforms from the same gene (see below).

Furthermore, we have observed the evident expansion of the ERM-like ancestor in vertebrates (Figure 1). Since the ERM homolog of Ciona emerged prior to the vertebrate clade, it appears that the first duplication of the vertebrate ERM sequence occurred after its divergence from Ciona. Subsequent expansion within this sub-family have led to the present existence of three related groups of proteins, ezrin, radixin and moesin, where the ezrin group is the most ancient. Such an expanded complement may be only common to the ERM proteins of vertebrates, because other metazoans have only one predicted ERM-like homolog [38-42]. Curiously, the increasing number of the ERM members occurred within the vertebrate clade paralleled with the evolutionary complication of the organism. Because of the diverse important functions of the ERM proteins [17,18], it would be important to understand how

these proteins are evolved and their functions coordinated.

Evolution of the functionally important residues in merlin. Although initial identification of proteins by sequence similarities does not yield a clear indication of their respective functions, analysis of specific conserved regions and residues may provide important information on their putative functional characteristics. We conducted pairwise sequence comparison among all obtained sequences of the ERM family and identified three regions of interest (Figure 2). First, in spite of sequence similarities in the N-terminal domain between the merlin and ERM proteins, merlin lacks a well-defined C-terminal actin-binding domain found in the ERM proteins [7,43-45]. Sequence comparison of the C-terminal region identified a noncontiguous stretch of 25 amino-acid residues, including the actin-binding site that are reliably aligned among all predicted ERM proteins with the exception of the so-called unnamed protein 2 of *Tetraodon nigroviridis*) (Figure 2A). According to the phylogenetic tree, the unnamed protein 2 is classified within the radixin group (Figure 1) and its sequence shows visible differences from other radixin proteins only at the C-terminus. The reason for such sequence variability is presently not known. It may be due to inaccuracy in sequence assembly from the scaffold. Alternatively, the unnamed 2 protein of *Tetraodon nigroviridis* may have a unique characteristic and will be of considerable interest for functional comparison with other radixin proteins.

Second, LaJeunesse et al. [46] previously identified in the N-terminal domain seven functionally important amino-acid residues (^{170}YQMTPEM^{177}), called the "Blue Box," that are identical in the human and *Drosophila* merlin proteins, but divergent from the ERM proteins. Sequence comparison revealed a more conserved pattern of this Blue Box region. All seven amino-acid residues of the Blue Box were found to be identical in the merlin sequences from vertebrates, fruit flies, and honey bee (Figure 2B); however, several amino-acid substitutions were found in those of worms, fishes and Ciona. The most interesting substitutions were from ^{174}ThrProGlu^{176} to ^{174}SerAlaAsp^{176}, found in the merlin-like proteins from *Caenorhabditis*. It is worth mentioning that the methione residue at position 177 in the Blue Box is conserved among all merlin proteins, but not in the ERM proteins. These results further corroborate the functional importance of these amino acids in the Blue Box [46].

Third, similar to the ERM proteins, the subcellular localization and intra- and inter-molecular

association of merlin are affected by phosphorylation [13,22-24,26,27,47]. In addition, phosphorylation also modulates the ability of merlin to suppress cell growth. Two phosphorylation sites have been mapped to the Ser^{518} and Thr^{576} residues in the merlin protein. Phosphorylation on the Ser^{518} residue has been shown to modulate the ability of merlin to form intramolecular associations and to bind to critical effectors important for growth suppression [27]. Phosphorylation on the Thr^{576} residue, however, has no effect on merlin's functional activity. In contrast, phosphorylation at the analogous residue is important for the function of the ERM proteins [45,48-50]. Sequence alignment shows that the Ser^{518} residue is conserved across all merlin proteins from different taxons with the exception of the fruit fly and worm, which contain a related threonine residue at the corresponding position. Since both the serine and threonine residues can be phosphorylated, we suggest that the corresponding threonine residue in the fly and worm merlin proteins may act as the phosphorylation site.

Gutmann et al. previously showed that mutations clustered in the predicted α–helical region did not affect merlin function, whereas those in either the N- or C-terminus of the peptide rendered merlin inactive as a negative growth regulator [20,21]. Specifically, five naturally occurring missense mutations L64P, K79E, E106G, L535P and Q538P were found to inactivate merlin function. Interestingly, we have found that the Leu^{64} and Lys^{79} residues are conserved among the merlin sequences from various organisms (Figure 2C). Also, the Glu^{106}, Leu^{535}, and Gln^{538} residues are similarly conserved within the merlin proteins of the Chordata group. These results highlight the general importance of these amino-acid residues for merlin function. In addition, we have also found that the glutamic acid residue at position 204 is conserved among all merlin proteins, but the corresponding amino acid at this position is variable in the ERM proteins (Figure 2B). Similarly, the isoleucine residue at position 546 is conserved among all merlin proteins, while a leucine amino acid is present at the corresponding position in the entire ERM group (Figure 2A). Furthermore, an amino-acid insertion between the residues 396 and 397 of the human merlin sequence was found in all ERM proteins but not in any merlin proteins. Collectively, it will be interesting to examine whether mutations in these residues could affect protein function.

Exon-intron structural evolution of the merlin gene. Recent progress in automated computational analysis of partially and completely sequenced genomes using gene prediction method together with the analysis of expressed sequence tag (EST) has provided considerable opportunity not only to describe the novel genes but also their exon-intron structures. Such an approach also allows examining the presence of different splicing variants/isoforms. To examine the evolution of the exon-intron structure, we assembled all available *NF2* gene-related sequences from different taxa. Using the sequences of proteins, mRNAs, and combined contigs (http://www.tigr.org/tdb/e2k1/bma1/), we have established the structure of the merlin-like gene for *Brugia malayi* which consisting of 12 exons and 11 introns (Figure 3). Analogously, the *NF2* homolog in *Caenorhabditis elegans* contains 11 exons and 10 introns. Intriguingly, the two *NF2*-like sequences nfm-1a and nfm-1b of *Caenorhabditis elegans* differ from each other only by the sequence of the last exon (Figure 3), predicting that the two above-mentioned merlin-like proteins erm-1a and erm-1b (Figure 1) represent protein isoforms.

As shown in Figure 3, the general arrangement of the merlin gene structure is conserved among mammalian species, especially at the region that encodes the N-terminal domain, albeit the number of exons may differ a little. The human *NF2* gene consists of 17 exons and spans about 95 kb of DNA [5,6,51,52]. *NF2* transcripts undergo alternative splicing, generating multiple isoforms [52-59]. Isoform I, missing exon 16, and isoform 2, containing all 17 exons, are the two predominant species. As the result of alternative splicing, isoform 1 encodes a 595 amino-acid protein. Isoform 2 differs from isoform 1 only at the C-terminus. Insertion of exon 16 into the mRNA provides a new stop codon, resulting in a 590 amino-acid protein that is identical to isoform 1 over the first 579 residues. Because of the presence of a long 3' untranslated region, the longest *NF2* isoform I RNA, containing the sequence from all 17 exons, is about 6.1 kb [52]. The merlin genes of R*attus norvegicus* and *Canis familiaris* contain 16 exons, whereas those of *Mus musculus* and *Pan troglodytes* have 15 exons. In addition, alternative spliced merlin isoforms have been found in the rodent species [60]. On the contrary, the structure of the merlin genes of *Gallus gallus* and *Fugu rubripes* are arranged differently from those of mammalian species, with 14 exons spreading over much shorter DNAs of only about 25 kb and 12.3 kb, respectively (Figure 3). In spite of the presence of 16 exons and the size of transcript

similar to those found in some vertebrates, the merlin-like gene of *Ciona intestinalis* is relatively small with only about 4.3 kb. This tendency towards reduction of intron length and number continues to be seen in the worm and particularly, in the insect. The merlin-like gene of *Caenorhabditis elegans*, consisting of 11 exons, spans about 4.7-kb DNA and that of *Brugia malayi*, containing 12 exons, is about 5.5 kb in length. The merlin gene of *Drosophila melanogaster* and the merlin-like gene of *Apis mellifera* are only about 2.9 kb, the smallest among the merlin clade, and consist of 5 and 8 exons, respectively (Figure 3).

Unlike the sizes and structures of the merlin genes, the lengths of the merlin proteins and transcripts have not been changing very much during evolution (Figure 3). Moreover, several functionally important regions of the merlin protein also remain conserved. Since the merlin homolog of the insect emerged after derivation from that of the worm, which was more ancient from the common ancestor (Figure 1), it appears that decreasing in gene size and exon number occurred specifically within the insect group. This branch of merlin evolution is likely to develop independently and in the opposite direction from those more recently developed merlin proteins of Chordata. Parallel evolution towards increasing merlin gene size and exon number between the worm and Chordata appears to be less likely.

It is evident that the genome of the insect is more complicated than that of the worm. Thus, the simplification of the merlin gene structure in the insect is unique and may have a functional significance. This may explain the lack of splicing variants in the insects, in contrast to those merlin isoforms found in mammals [52,54-59,61,62] and in *Caenorhabditis elegans* as we have predicted in this study.

Conclusion

We have conducted the phylogenetic analysis of merlin diversity across metazoan genomes using the experimentally annotated and predicted sequences in conjunction with bioinformatics tools. We show that the merlin proteins have a monophyletic origin with the root in early metazoan. We have also established the expansion of the ERM-like ancestors within the vertebrate clade that occurred after its separation from Urochordata. Several potentially important sites that are conserved among all merlin proteins but divergent in the ERM members have been identified. Analysis of the evolution of the

merlin gene structure reveals the existence of common splicing variants in human and *Caenorhabditis elegans*. Taken together, our results have important implications on the evolution of the merlin proteins and their possible functional variability in different taxons.

Methods

BLAST search. Initial sequences of genes and proteins of interest from various organisms were identified from the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov/BLAST) using the BLAST algorithm [63]. We then searched the desirable sequences across genomic databases of completely or partially sequenced genomes available at The Sanger Institute (http://www.sanger.ac.uk/DataSearch) and The Institute for Genomic Research (TIGR) (http://tigrblast.tigr.org/tgi/). Also, we investigated other available sequence databases that contain information for specific organisms. The sources of sequences of the predicted or experimentally annotated merlin and ERM proteins are summarized in Table 1.

To obtain the entire amino-acid sequence of an annotated protein, we used UniProt from Universal Protein Resource (http://www.ebi.uniprot.org/index.shtml). The erythrocyte membrane proteins 4.1 sequences of *Homo sapiens* (GenBank: CAI21970), *Mus musculus* (GenBank: NP_001006665), and *Danio rerio* (GenBank: AAQ97985) were also included in the analysis as an outgroup. Because of the presence of many non-conservative and large introns in the genes of interest, we conducted BLAST search using TBLASTN alignment algorithm in the cases where no protein sequences were available.

Alignments and phylogeny. The Clustal X program [64] was used to align the characterized or predicted protein sequences from different species. Phylogenetic analysis was carried out using the MEGA2.1 program [37].

List of Abbreviations

NF2 - the *neurofibromatosis type 2* gene

ERM - ezrin, radixin, and moesin

NF2 - Neurofibromatosis type 2

FERM - 4.1, ezrin, radixin, and moesin

TIGR - The Institute for Genomic Research

EST, expressed sequence tag

NCBI - National Center for Biotechnology Information

Authors' Contributions

KG and AB carried out the phylogenetic analysis of merlin diversity across metazoan genomes and drafted the manuscript. EMA and LVO helped with the design of the study and preparation of data for the figures. LSC is the principal investigator of the project, participated in the design, coordination, and writing of the manuscript. All authors read and approved the final manuscript

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Table 1. The list of the predicted and experimentally annotated merlin and ERM proteins included in this study. Numbers indicated in bold letters are not GenBank Accession Numbers, but were obtained from genome sequencing projects..

Species	Proteins	GenBank Acession No.	*Related Resources or* Sequencing Projects
Homo sapiens	merlin (NF2) ezrin radixin moesin	P35240 P15311 P35241 P26038	*http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=9558*
Pan troglodytes	similar to NF2	XP_515061	*http://www.hgsc.bcm.tmc.edu/projects/chimpanzee/*
Papio anubis	merlin	AAO23133	*http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=12965*
Bos Taurus	ezrin	NP_776642	*http://www.hgsc.bcm.tmc.edu/projects/bovine/*
Sus scrofa	radixin moesin	P26044 P26042	*http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=pig*
Canis familiaris	similar to NF2	XP_534729	*http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=dog*
Oryctolagus cuniculus	ezrin	Q8HZQ5	*http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=12818*
Mus musculus	ezrin radixin merlin	P26040 NP_033067 NP_035028	*http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=mouse*
Rattus norvegicus	ezrin NF2	NP_062230 XP_341249	*http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=rat*
Gallus gallus	ezrin radixin merlin	NP_990216 Q9PU45 NP_989828	*http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=g_gallus*
Xenopus laevis	unknown protein	**AAH77822**	*http://www.xenbase.org/*
Danio rerio	nf2a moesin	NP_998116 NP_001004296	*http://www.ensembl.org/Danio_rerio/*
Fugu rubripes	radixin moesin merlin	**FRUP00000132603** **FRUP00000156313** **FRUP00000136298**	*http://genome.jgi-psf.org/*
Tetraodon nigroviridis	unnamed protein 1 unnamed protein 2	CAG08868 CAG08250	*http://www.ensembl.org/Tetraodon_nigroviridis/*
Ciona intestinalis	erm-like merlin-like	**ci0100149701** **ci0100130636**	*http://genome.jgi-psf.org/*
Ciona savignyi	merlin-like		*http://www.broad.mit.edu/ftp/*
Biomphalaria glabrata	erm-like	**AAK61353**	*http://biology.unm.edu/biomphalaria-genome/*
Lytechinus variegates	moesin	**P52962**	*http://www.hgsc.bcm.tmc.edu/projects/seaurchin/*
Apis mellifera	similar to schwannomin	**XP_392673**	*http://racerx00.tamu.edu/PHP/bee_search.php*
Drosophila melanogaster	merlin moesin	**Q24564** P46150	*http://flybserver.gen.cam.ac.uk:7081/*
Drosophila yakuba	merlin-like	predicted in this work	*http://genome.wustl.edu/blast/client.pl*
Anopheles gamblae	merlin-like fragment	**EAA07087**	*http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=mosquito*
Caenorhabditis elegans	erm1a erm1b nfm 1a nfm 1b	AAB37643 AAB37642 NP_498335 NP_498336	*http://www.wormbase.org/*
Caenorhabditis briggsae	erm-like nfm1	**BP:CBP03133** **BP:CBP05025**	*http://www.wormbase.org/*
Caenorhabditis remanie	merlin-like erm-like	predicted in this work	*http://genome.wustl.edu/blast/client.pl*
Brugia malayi	merlin-like	316.m00022	*http://www.tigr.org/tdb/e2k1/bma1/*
Schistosoma japonicum	JF2	**AAB49033**	*http://www.nhm.ac.uk/hosted_sites/schisto/*
Taenia saginata	myosin-like	**CAA65728**	*http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=protein*
Echinococcus multilocularis	EM10	**A45620**	*http://www.sanger.ac.uk/Projects/Echinococcus/*
Echinococcus granulosus	EG10	**CAA82625.1**	
Phanerochaete chrysosporium	---		*http://genome.jgi-psf.org/whiterot1/whiterot1_home.html*
Aspergillus flavus	---		*http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=a_flavus*
Arabidopsis thaliana	---		*http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=arab*
Oryza sativa	---		*http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=rice*
Trypanosoma brucei	---		*http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=t_brucei*
Cryptosporidium parvum	---		*http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=c_parvum*

FIGURE LEGENDS

Figure 1. The neighbor-joining tree of the ERM family. The diagram illustrates the basic resolution of the ERM-family members into two major clades, merlin and ERM. Bootstrap support values are shown above each node. Color-shaded boxes denote different subgroups of the ERM clade in vertebrates, which appeared after the expansion of the ERM-like ancestor occurring after its separation from Urochordata (*Ciona intestinalis*). The *Tetraodon nigroviridis* "unnamed protein 1 and 2" sequences (GenBank Accession No. CAG08868 and CAG08250, respectively) and the *Xenopus laevis* "unknown protein" sequence (GenBank Accession No. AAH77822) were grouped based on their homology with the merlin or ERM sequences.

Figure 2. Sequence alignments of functionally important regions in the merlin and ERM proteins. Databank resources for the ERM-family proteins listed in Table 1 were used in the analysis, and only typical representatives from each group displayed. (A) Alignment of the N-terminal domain containing the "Blue Box" (^{170}YQ-MTPEM^{177}) [46] and the amino-acid residue 204, conserved among the merlin proteins but divergent in the ERM proteins. (B) Comparison of the C-terminal region including the potential actin-binding site and two other predicted significant residues. (C) Conservation of functionally important residues including the phosphorylation site in the merlin group.

Figure 3. Schematic diagram of the exon-intron structures of the merlin genes from various metazoans. The horizontal line depicts the merlin gene with its size indicated in bp (base pairs) on the right. The upright boxes represent exons. The lengths of the available merlin mRNA sequences in the database are shown in nucleotides (nt) and the lengths of the predicted merlin proteins are also indicated in amino acids (aa). The indicated human *NF2* mRNA refers to the longest, full-length transcript identified, which contains a long 3' untranslated region [52]. Via alternative splicing, two major human *NF2* isoforms I and II are produced and their protein lengths are shown with that of isoform I indicated in the parenthesis. It should be noted that Northern blot analysis detected the rat and mouse *NF2* mRNAs of about 4.5 kb, indicating that the sizes of the rodent *NF2* mRNAs shown are not full-length. The asterisk (*) indicates that the exon-intron structure of *Brugia malayi* was predicted from this study.

Figure 1.

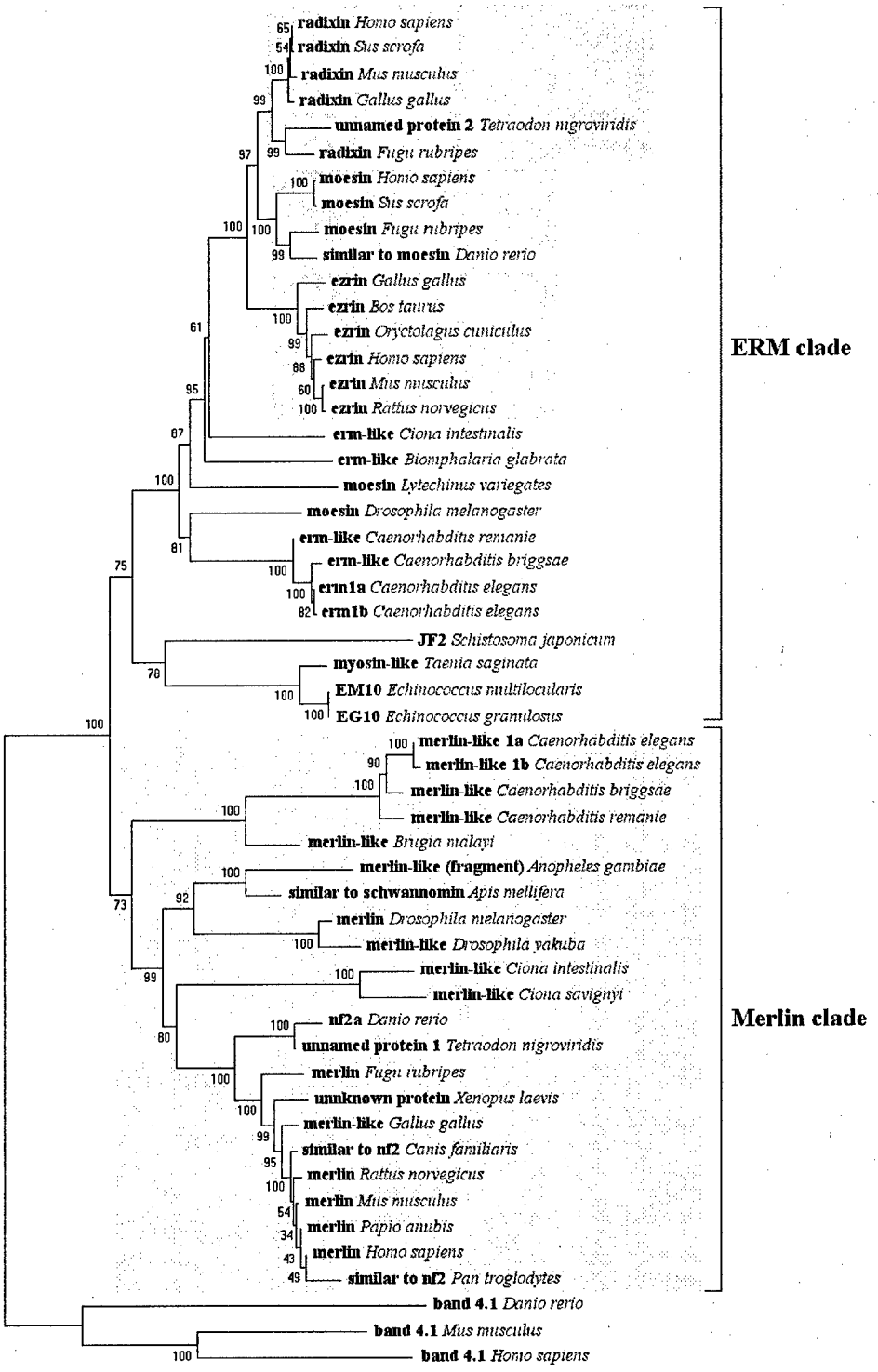

radixin Homo sapiens
radixin Sus scrofa
radixin Mus musculus
radixin Gallus gallus
unnamed protein 2 Tetraodon nigroviridis
radixin Fugu rubripes
moesin Homo sapiens
moesin Sus scrofa
moesin Fugu rubripes
similar to moesin Danio rerio
ezrin Gallus gallus
ezrin Bos taurus
ezrin Oryctolagus cuniculus
ezrin Homo sapiens
ezrin Mus musculus
ezrin Rattus norvegicus
erm-like Ciona intestinalis
erm-like Biomphalaria glabrata
moesin Lytechinus variegates
moesin Drosophila melanogaster
erm-like Caenorhabditis remanie
erm-like Caenorhabditis briggsae
erm1a Caenorhabditis elegans
erm1b Caenorhabditis elegans
JF2 Schistosoma japonicum
myosin-like Taenia saginata
EM10 Echinococcus multilocularis
EG10 Echinococcus granulosus
merlin-like 1a Caenorhabditis elegans
merlin-like 1b Caenorhabditis elegans
merlin-like Caenorhabditis briggsae
merlin-like Caenorhabditis remanie
merlin-like Brugia malayi
merlin-like (fragment) Anopheles gambiae
similar to schwannomin Apis mellifera
merlin Drosophila melanogaster
merlin-like Drosophila yakuba
merlin-like Ciona intestinalis
merlin-like Ciona savignyi
nf2a Danio rerio
unnamed protein 1 Tetraodon nigroviridis
merlin Fugu rubripes
unnknown protein Xenopus laevis
merlin-like Gallus gallus
similar to nf2 Canis familiaris
merlin Rattus norvegicus
merlin Mus musculus
merlin Papio anubis
merlin Homo sapiens
similar to nf2 Pan troglodytes
band 4.1 Danio rerio
band 4.1 Mus musculus
band 4.1 Homo sapiens
ERM clade
Merlin clade

Figure 2A.

	Blue Box	204
H. sapiens **merlin**	INLYQ-MTPEMWEERITAWYAEHRGRARDEAEMEYLK	209
C. familiaris **similar to nf2**	INLYQ-MTPEMWEERITAWYAEHRGRARDEAEMEYLK	318
M. musculus **merlin**	INLYQ-MTPEMWEERITAWYAEHRGRARDEAEMEYLK	209
G. gallus **merlin-like**	INLYQ-MTPEMWEERITAWYAEHRGRARDEAEMEYLK	209
X. laevis **unknown protein**	INLYQ-MTPEMWEERITAWYAEHRGRTRDEAEMEYLK	209
D. rerio **nf2a**	LMQYQ-MTPDMWEEKITAWYAEHRNITRDEAEMEYLK	201
F. rubripes **merlin**	INLYQ-MTAEMWEERITACYAEHRGRTRDEAEMEYLK	170
C. intestinalis **merlin-like**	RDQFQSVTGEMWETQITSWYAQHHGLTRDEAELEYLK	207
C. savignyi **merlin-like**	IDQYQSVTGQMWEAQITPWYAGHHGLTRDEAELEYLK	179
A. gambiae **fragment of merlin**	--QYQ-MTPQMWEERIKTWYADHRGMSRDEAEMEYLK	34
A. mellifera **similar to schwannomin**	IDQYQ-MTPEMWEDRIKIWYADHRGMSRDEAEMEYLK	201
D. melanogaster **merlin**	TDQYQ-MTPEMWEERIKTWYMDHEPMTRDEVEMEYLK	203
C. elegans **merlin-like**	IDQYD-MSADMWRDRIKRWWSRNAGQSREEAELEYLR	203
C. briggsae **merlin-like**	IDQYD-MSADMWRDRIKRWWSRNAGQSREEAELEYLR	203
B. malayi **merlin-like**	IKQYD-MTPOMWEERIKRWWINNSGQSREDAEMEYLR	196
H. sapiens **ezrin**	MDQHK-LTRDQWEDRIQVWHAEHRGMLKDNAMLEYLK	193
B. taurus **ezrin**	MDQHK-LTRDQWEDRIQVWHAEHRGMLKDSAMLEYLK	193
G. gallus **ezrin**	MDQHK-LSRDQWEERIQVWHAEHSGMLKENAMLEYLK	193
H. sapiens **radixin**	LEQHK-LTKEQWEERIQNWHEEHRGMLREDSMMEYLK	193
G. gallus **radixin**	LEQHK-LTKEQWEERIQNWHEEHRGMLREDSMMEYLK	193
T. nigroviridis **unnamed protein 2**	LEQHK-LTKEQWEERIQTWHEEHRSMLREDAMMEYLK	189
H. sapiens **moesin**	LEQHK-LNKDQWEERIQVWHEEHRGMLREDAVLEYLK	193
F. rubripes **moesin**	LDQHK-LNKDQWEERIQVWHEEHKGMMREESMMEYLK	189
C. intestinalis **erm-like**	YEQHK-MTKEQWEERIQTWHCEHGSMTREDAMIEYLK	217
B. glabrata **erm-like**	YDQHK-LTKEQWEERIKSWYAEHKAMLREDAMIEYLK	194
L. variegates **moesin**	IEQHK-MTKEQWYERVSNWHQEHLSLSKEDAITEYMK	192
C. elegans **erm-like 1a**	LGQFK-LNSEEWERRIMTWWADHRATTREQAMLEYLK	194
C. briggsae **erm-like**	LGQFK-LNSEEWERRIMTWWADHRATTREQAMLEYLK	189
D. melanogaster **moesin**	IDQHK-MSKDEWEQSIMTWWQEHRSMLREDAMMEYLK	194
T. saginata **myosin-like**	KDQYD-QTDEQWFDRIVTYYKDHHDMSREDAMVQYLQ	195
E. multilocularis **EM10**	-EQYD-QTDEQWYERIIAYYKDHHDMSREDAMVQYLQ	195

Figure 2B.

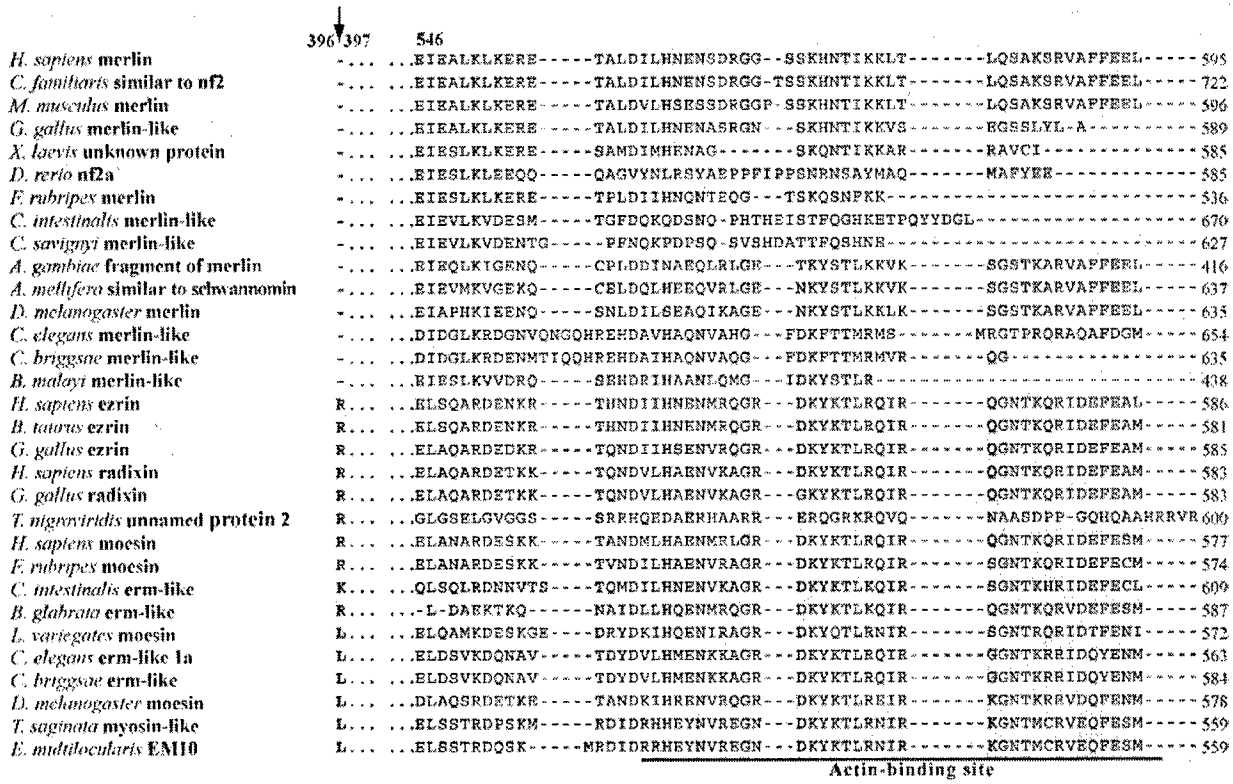

396 397 546
H. sapiens merlin
C. familiaris similar to nf2
M. musculus merlin
G. gallus merlin-like
X. laevis unknown protein
D. rerio nf2a
F. rubripes merlin
C. intestinalis merlin-like
C. savignyi merlin-like
A. gambiae fragment of merlin
A. mellifera similar to schwannomin
D. melanogaster merlin
C. elegans merlin-like
C. briggsae merlin-like
B. malayi merlin-like
H. sapiens ezrin
B. taurus ezrin
G. gallus ezrin
H. sapiens radixin
G. gallus radixin
T. nigroviridis unnamed protein 2
H. sapiens moesin
F. rubripes moesin
C. intestinalis erm-like
B. glabrata erm-like
L. variegates moesin
C. elegans erm-like 1a
C. briggsae erm-like
D. melanogaster moesin
T. saginata myosin-like
E. multilocularis EM10
Actin-binding site

Figure 2C.

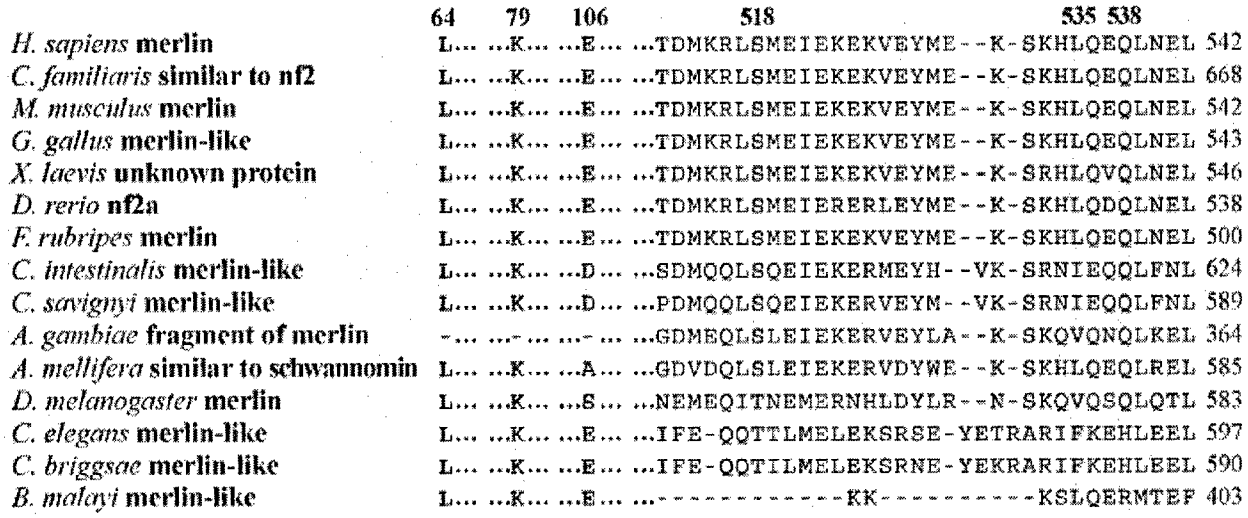
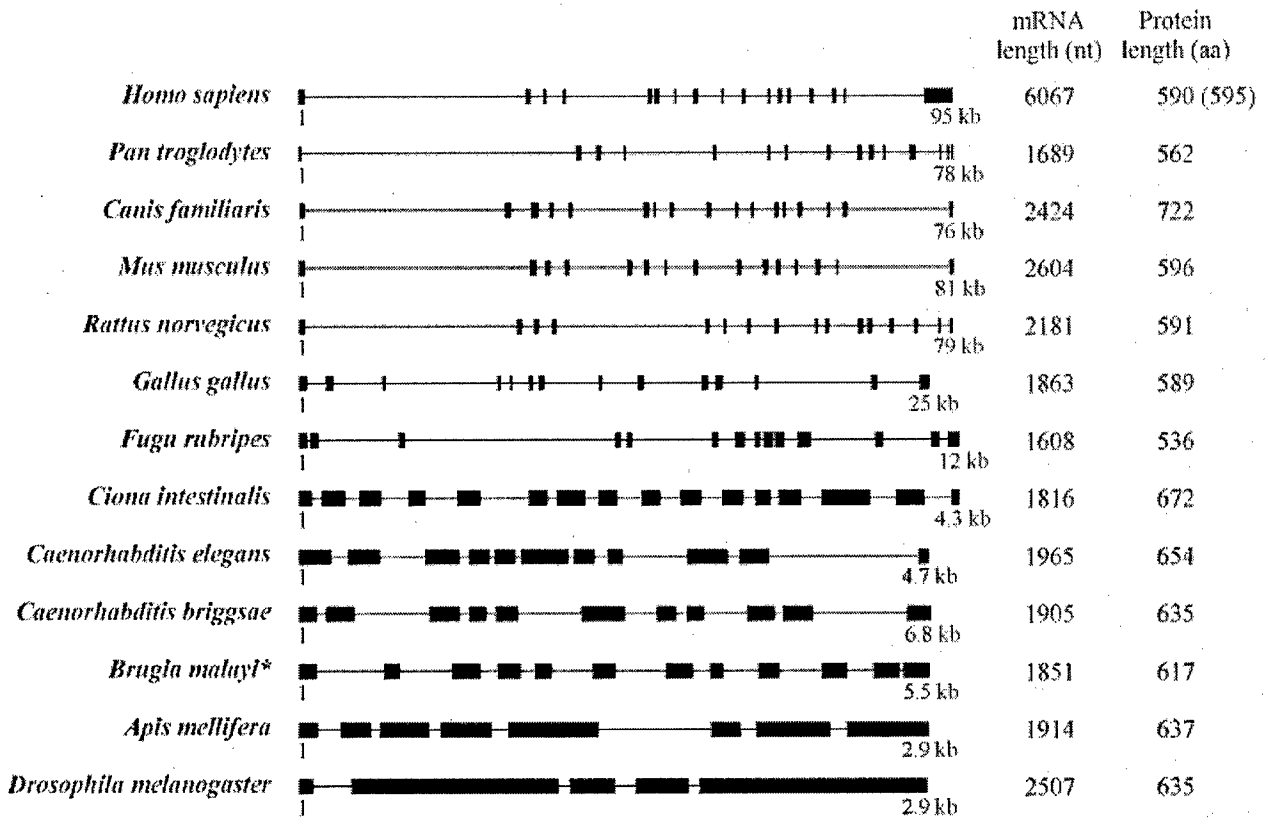

64 79 106 518 535 538
H. sapiens merlin L... ...K... ...E... ...TDMKRLSMEIEKEKVEYME--K-SKHLQEQLNEL 542
C. familiaris similar to nf2 L... ...K... ...E... ...TDMKRLSMEIEKEKVEYME--K-SKHLQEQLNEL 668
M. musculus merlin L... ...K... ...E... ...TDMKRLSMEIEKEKVEYME--K-SKHLQEQLNEL 542
G. gallus merlin-like L... ...K... ...E... ...TDMKRLSMEIEKEKVEYME--K-SKHLQEQLNEL 543
X. laevis unknown protein L... ...K... ...E... ...TDMKRLSMEIEKEKVEYME--K-SRHLQVQLNEL 546
D. rerio nf2a L... ...K... ...E... ...TDMKRLSMEIERERLEYME--K-SKHLQDQLNEL 538
F. rubripes merlin L... ...K... ...E... ...TDMKRLSMEIEKEKVEYME--K-SKHLQEQLNEL 500
C. intestinalis merlin-like L... ...K... ...D... ...SDMQQLSQEIEKERMEYH--VK-SRNIEQQLFNL 624
C. savignyi merlin-like L... ...K... ...D... ...PDMQQLSQEIEKERVEYM--VK-SRNIEQQLFNL 589
A. gambiae fragment of merlin -... ...-... ...-... ...GDMEQLSLEIEKERVEYLA--K-SKQVQNQLKEL 364
A. mellifera similar to schwannomin L... ...K... ...A... ...GDVDQLSLEIEKERVDYWE--K-SKHLQEQLREL 585
D. melanogaster merlin L... ...K... ...S... ...NEMEQITNEMERNHLDYLR--N-SKQVQSQLQTL 583
C. elegans merlin-like L... ...K... ...E... ...IFE-QQTTLMELEKSRSE-YETRARIFKEHLEEL 597
C. briggsae merlin-like L... ...K... ...E... ...IFE-QQTILMELEKSRNE-YEKRARIFKEHLEEL 590
B. malayi merlin-like L... ...K... ...E... ...----------KK----------KSLQERMTEF 403

Figure 3.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Protein length (aa)
Homo sapiens
95 kb
6067
590 (595)
Pan troglodytes
78 kb
1689
562
Canis familiaris
76 kb
2424
722
Mus musculus
81 kb
2604
596
Rattus norvegicus
79 kb
2181
591
Gallus gallus
25 kb
1863
589
Fugu rubripes
12 kb
1608
536
Ciona intestinalis
4.3 kb
1816
672
Caenorhabditis elegans
4.7 kb
1965
654
Caenorhabditis briggsae
6.8 kb
1905
635
Brugia malayi*
5.5 kb
1851
617
Apis mellifera
2.9 kb
1914
637
Drosophila melanogaster
2.9 kb
2507
635