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14. ABSTRACT The development of effective and safe therapeutics that can be used to combat the debilitating and incurable malignancies that are associated with neurofibromatosis type II (NF2) clearly requires a detailed understanding of the underlying molecular basis of this disease. NF2 is the gene that encodes the cytoskeletal tumor suppressor protein merlin, which is required for the development and stabilization of cell-cell contacts that are mediated by specialized cell surface structures called adherens junctions. Loss-of-function mutations of merlin are the hallmark of NF2, and these mutations disable its functions in controlling adherens junctions, provoking uncontrolled cell growth and leading to the development of ultimately lethal tumors, including schwannoma, meningioma, and mesothelioma. This award allowed us to perform a series of structural and biochemical studies that provide the foundation for developing small molecule therapeutic agents that bind and reactivate the tumor suppressor activity of mutated merlin.					
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1. Introduction

Loss-of-function mutations in the *neurofibromatosis-2* (*NF2*) gene lead to familial and sporadic neurological malignancies in man, specifically to schwannomas and meningiomas, and also to malignant mesothelioma in the lung. Furthermore, *Nf2* heterozygosity in mice leads to the development of a number of aggressive tumor types, indicating that merlin, the cytoskeletal protein encoded by the *NF2* gene, plays broad roles in harnessing tumorigenesis. Tragically, *NF2*-associated malignancies are essentially incurable and the overall outcome of Neurofibromatosis Type II (NF2) patients with these cancers remains dismal, despite aggressive therapy. Thus, there is a clear and immediate need for the development of new therapeutics, which can only come from a more thorough understanding of the molecular basis of these malignancies.

Merlin is required for the development and stabilization of specialized cell-cell contacts termed adhesion junctions. Loss of function mutations in merlin disables adherens junctions in primary Schwann cells, the principle target cell where cancer arises in NF2 patients. As a consequence of merlin loss, *NF2*-deficient cells display uncontrolled growth, loss of contact inhibition, and alterations in the expression of mitogenic signaling cell surface receptors. Notably, all of these phenotypes are suppressed by re-introduction of merlin into human or mouse *Nf2*-deficient Schwann cells, establishing merlin as a *bona fide* tumor suppressor.

Merlin is a member of the ERM (ezrin-radixin-moesin) family of proteins that provide essential links of the actin cytoskeleton to the cell membrane, and which play critical roles in the organization of epithelial cells in tissues. Crystal structures have thus far revealed that ERM proteins have a tripartite structure in their closed-clamp, inactive conformation. Here their *N*-terminal four-point-one-ezrin-radixin-moesin (FERM) domain interacts with a *C*-terminal actin-binding tail domain, and also with a central α -helical domain. Severing these intramolecular interactions, which can occur through phosphorylation and/or *via* binding to phospholipids, activates ERMs, allowing them to bind to their partners. The many missense mutations found in *NF2* are thought to break merlin head-tail interactions, thus opening up and inactivating merlin. Finally, merlin does not bind to actin through its *C*-terminus but rather, by unknown means, *via* its FERM domain.

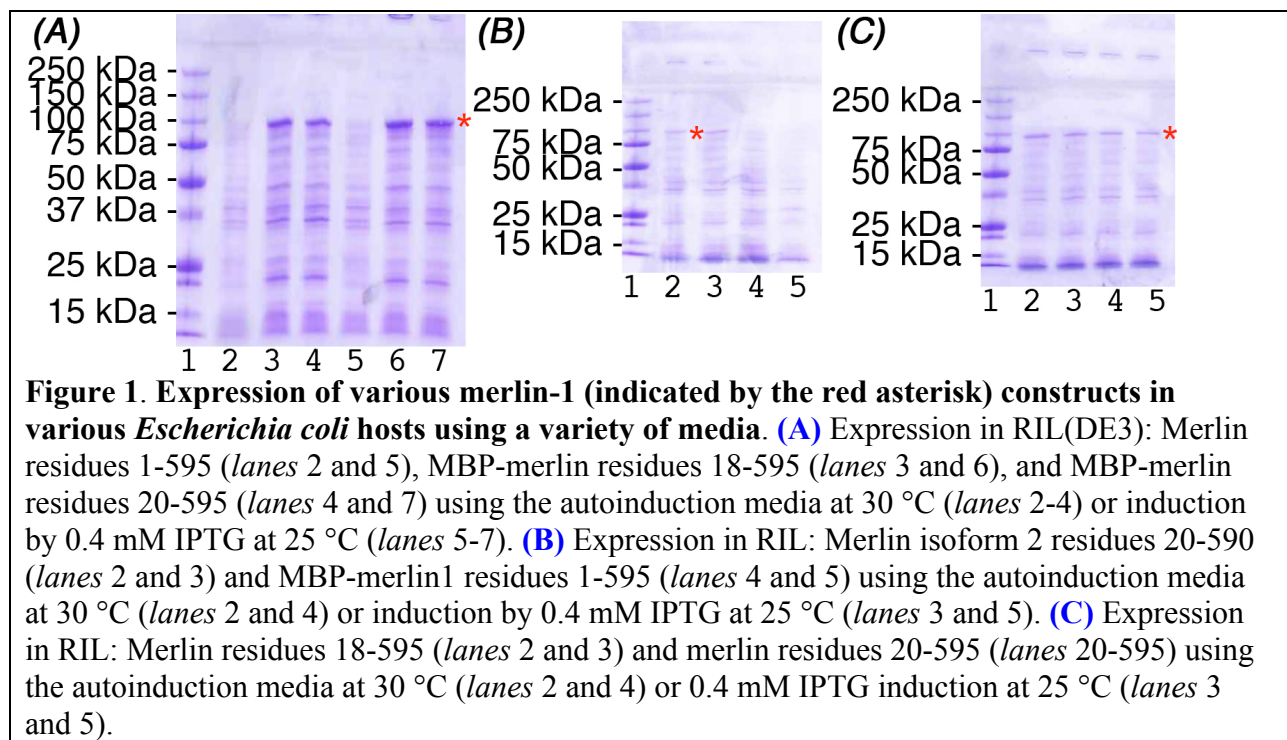
The goal of this DOD Neurofibromatosis New Investigator award is to gain mechanistic and structural information of merlin that provide the foundation for developing small molecule therapeutic agents that bind and reactivate the tumor suppressor activity of mutated merlin in patients suffering from neurofibromatosis.

2. Body

Aim 1: What is the crystal structure of the merlin tumor suppressor?

The first crucial step towards determining the full-length human high-resolution crystal structure of merlin is the generation of large amounts of homogeneous purified merlin, which often is not a trivial task and in this case is a challenge. We explored several expression hosts and constructs with different affinity tags. To keep this report focused, only the key results are shown.

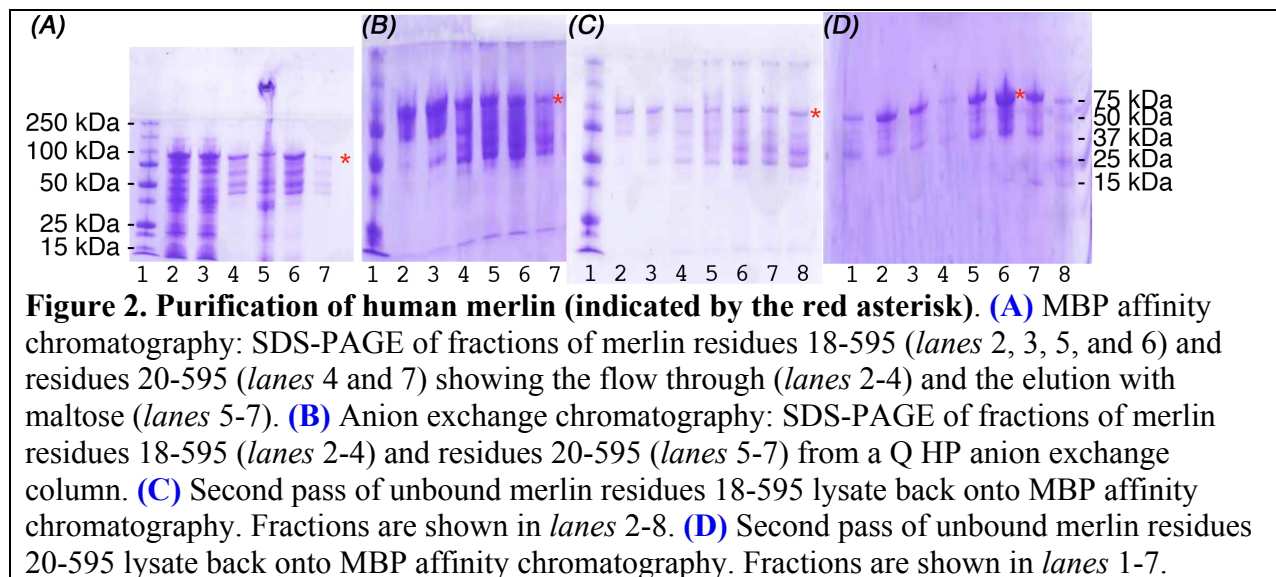
For full-length merlin production, we used several constructs for isoform 1, in particular all residues (1-595), constructs with the disordered *N*-terminus deleted (18-595 or 20-595) and also isoform 2 (residues 20-590). For expression in *Escherichia coli*, we used several strains, such as BL21(DE3), Rosetta, Origami, RIL, or RIL(DE3). Best success was accomplished as an MBP-fusion. Merlin did not express in BL21(DE3), Rosetta, or Origami (data not shown) but in RIL, or RIL(DE3). As is evident, amino-terminal truncation is crucial to obtain good expression (**Figure 1**).



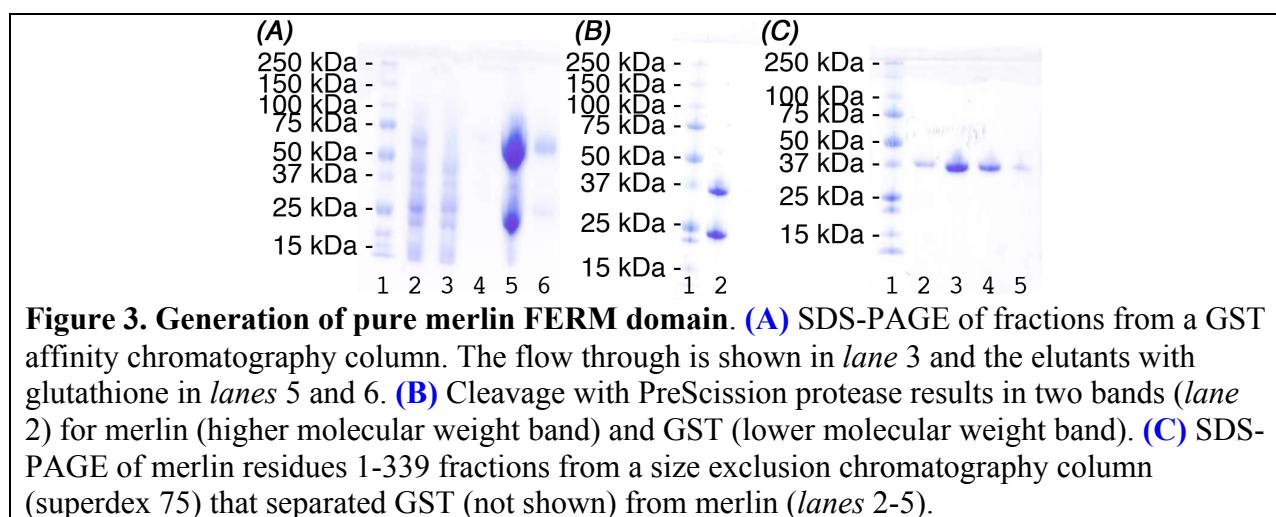
After obtaining good expression results (**Figure 1**), we proceeded with protein purification. A large amount of parameters were varied and tested and only the major results are presented which can be summarized in difficulties in purification to homogeneity. Despite a large number of tested chromatography columns, lower molecular weight impurities remain and binding to the amylose affinity column was incomplete (**Figure 2**).

To overcome the expression and purification difficulties described above, we proceeded with merlin (residues 1-595, 18-595, and 20-595) expression in *Pichia pastoris* using standard vectors

as well as vectors that secrete recombinant proteins due to an *N*-terminal peptide encoding *Saccharomyces cerevisiae* secretion signal. We generated constructs harboring a C-terminal cleavable tag comprising GFP for easy detection of expression followed by ten Histidine residues for easy purification.

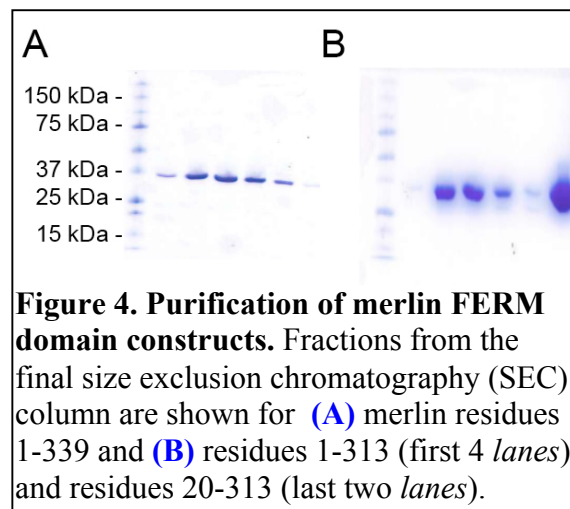


We also obtained a construct of merlin residues 1-339 from our colleague in our Department, Dr. Joseph Kissil, as a GST-fusion. Although we were able to obtain good expression in Rosetta, RIL, and RIL(DE3), the expressed protein was insoluble (data not shown). This was eventually overcome by lowering the expression temperature to 25 °C and resulted in pure protein (**Figure 3**).



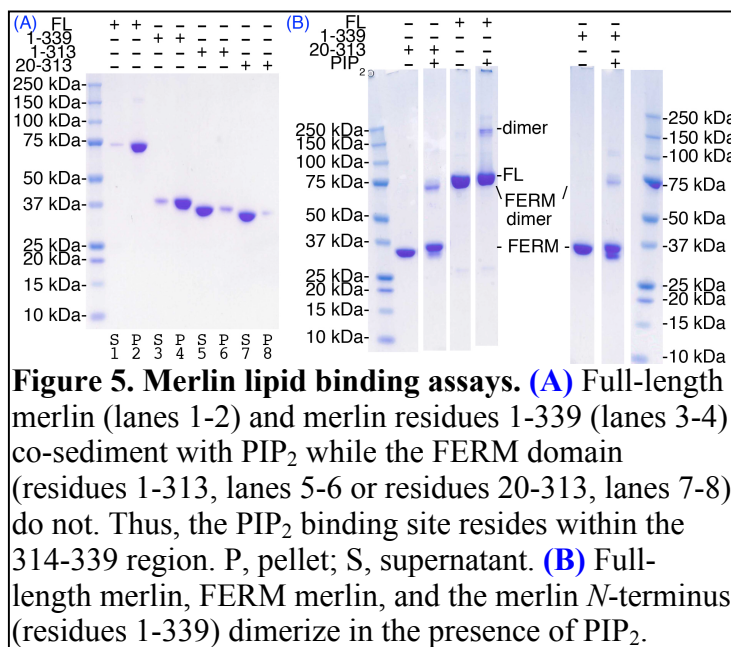
Obtaining merlin crystals that diffract X-rays to high resolution resulted more difficult than anticipated. We continued with our protein production and expressed of the FERM domain *N*-terminally fused to GST in *E. coli* BL21(DE3) cells and purified merlin using a GST affinity chromatography column (GE life sciences) followed by overnight PreScission protease cleavage

at 4 °C and a sizing chromatography column (Superdex 75; GE life sciences). Since ligands often aid crystallization, we bound phosphatidylinositol 4,5-bisphosphate (PIP₂) to our merlin proteins and were able to grow merlin FERM domain (residues 1-313) crystals in complex with a PIP₂ derivative having a short phosphoinositide chain [(CH₂)₈] that diffracted X-rays to about 2.5 Å Bragg spacings. We collected ten complete datasets and processed the X-ray diffraction data using autoPROC. We used the merlin FERM domain (PDB entries 3u8z and 1h4r) as search models for molecular replacement and we obtained clear solutions using MolRep. However, after crystallographic refinement using autoBuster, we were unable to identify any bound lipid bound.



To better identify the ligand-binding site that might aid full-length merlin crystallizations, we cloned, expressed, and purified several other merlin constructs to fully define the PIP₂ binding site (**Figure 4**). Consistent with our crystallographic results, merlin residues 1-313 or 20-313 showed only weak binding to PIP₂ in our lipid binding assays (**Figure 5**). Therefore, we screened further and identified a merlin construct (residues 1-339) that includes the FERM domain and an extended C-terminal α -helix that binds PIP₂ much more readily in the lipid sedimentation assay than the previous merlin constructs (**Figure 4**). This C-terminal α -helix harbors seven positively charged residues (314-DSLEVQQMKAKA**REEKARKQMERQRL**-339) that could mediate PIP₂ binding (**Figure 6**). We screened over 1,000 crystallization conditions at room temperature and at 4 °C and obtained small plate like crystals for the merlin/PIP₂-diC8 complex from 0.1 M HEPES (pH 7) and 8% (w/v) PEG 8,000 that diffracted X-rays to about 3 Å Bragg spacings (**Figure 7A**). While the crystal morphology was improved by increasing the concentration of PIP₂ to 300 μ M, these crystals diffracted only to about 8.5 Å and were highly mosaic (**Figure 7B**).

We collected X-ray diffraction data at SER-CAT beam lines BM22 and ID22 at the Advanced Photon Source at Argonne National Laboratory (Argonne, IL) and reduced the data using XDS as implemented in autoPROC. The data reduction statistics are provided in **Table 1**. Radiation damage and anisotropy prevented higher overall completeness. However, these crystals are twinned and the



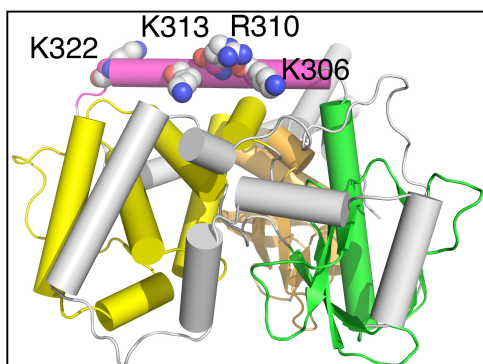


Figure 6. Location of our identified PIP₂ binding site.

Cartoon drawing of the full-length moesin structure, FERM subdomains colored spectrally and the PIP₂ binding α -helix in magenta showing the exposed charged residues that correspond to merlin residues K322, R326, K329, and R338.

crystallization attempts were therefore unsuccessful. We next expressed and purified the S518D phosphomimetic merlin mutant but this mutant was also unstable (data not shown). Significantly, full-length A585W/R585K mutant merlin was stable to concentrations of about 10 mg/ml (**Figure 5**) and bound PIP₂ readily (**Figure 5**). Thus, the lipid-binding site in merlin is accessible for PIP₂ binding.

Which conformation of merlin is the tumor suppressive active form is hotly debated [1-4]. Indeed, merlin has long been thought to be similar in structure to ERMs yet it is known to be different in actin binding and, unlike other ERMs, the ostensibly ‘closed’ clamp conformer of merlin has long been thought to represent its tumor suppressor-active state which has been challenged recently [4, 5]. Instead, a hierarchy of closure was suggested with the A585W/R588K mutant being the most tightly closed, followed by phosphomimetic S518D, wild type, and phospho-deficient S518A, and finally a truncation of the last two residues or merlin isoform 2 which are fully open. Further, wild type, S518D, and S518E merlin have been suggested to be monomeric [6]

diffraction is highly anisotropic (**Figure 7**).

Armed with the success of crystallizing the merlin N-terminus in complex with PIP₂, we returned to full-length merlin expression in *E. coli* BL21(DE3) cells at 16 °C. Our initial constructs contained a GST-tag but purified full-length merlin protein was susceptible to aggregation during concentrating the protein to over 3 mg/ml and

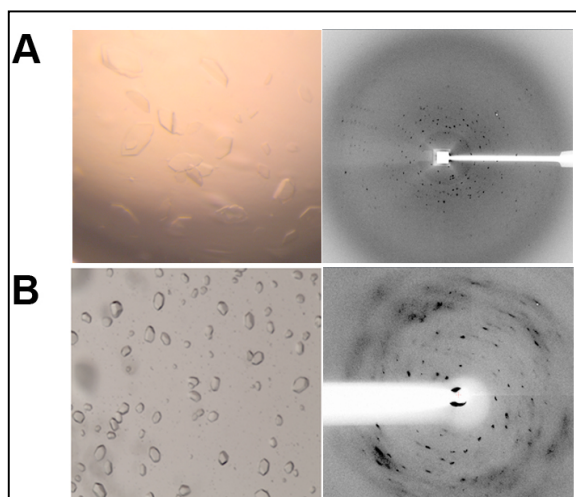


Figure 7. Crystals and X-ray diffraction of the merlin FERM domain [residues 1-339] in complex with PIP₂. (A) Crystals of FERM domain and 200 μ M PIP₂-diC8 complex appeared in 2 days and they diffracted X-rays to 3.0 Å at SER-CAT beam line BM22 at APS. (B) Merlin FERM domain and 300 μ M PIP₂-diC8 complex crystals appeared in 3 days and they diffracted X-rays to 8.5 Å at SER-CAT beam line ID22 at APS.

Table 1. X-ray data and reduction statistics for merlin N-terminus in complex with diC8-PIP₂ crystals.

Space group	C2 ₁
Cell dimensions	86 Å, 97 Å, 99 Å, 99°
Resolution	99 Å – 3.23 Å
Last shell	3.38 Å – 3.23 Å
R-merge	0.123 (0.217)
Average I/ σ (I)	22.1 (2.4)
Completeness	0.951 (0.952)
Multiplicity	3.8 (3.3)

yet our full-length A585W/R588K merlin mutant is clearly a dimer in the presence or absence of PIP₂ (**Figure 8**). It was suggested that monomeric merlin binds PIP₂ 5-fold tighter than the merlin FERM domain and that the closed and more open merlin conformers bind PIP₂ [6] which seems contradictory and probably resulted from the fact that the used PIP₂ concentrations were 30-fold above the critical micelle concentration and resulted in precipitation and perhaps no PIP₂ bound to the soluble protein fraction. We hypothesize that merlin binds PIP₂ in its closed conformation.

Aim 2: What are the effects of heterotypic interactions with ezrin on the merlin structure?

Due to the unexpected hurdles with **Aim #1**, we were not able to devote as much time to **Aim #2** as hoped and despite of significant efforts we were unable to co-purify the heterodimer for structural studies. We did however obtain expression for the individual domains (**Figure 9**) that we were able to purify and will attempt to complete this aim when possible.

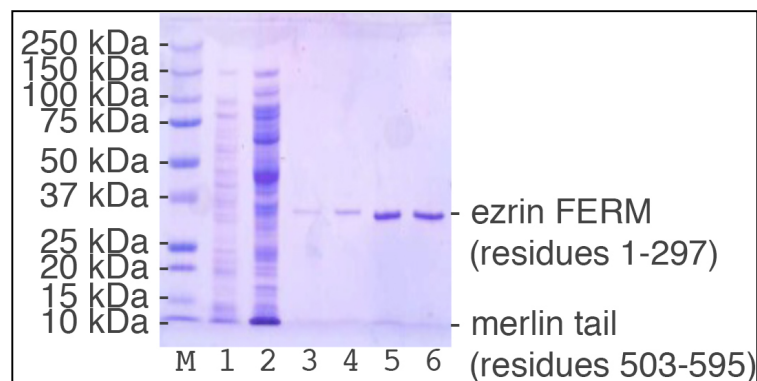


Figure 9. Co-purification of ezrin FERM domain and merlin tail domain. 10%-15% SDS-PAGE gradient gel after passage through Ni affinity chromatography column. *Lane 1*, flow through; *lane 2*, buffer containing most unbound and untagged merlin tail; *lanes 3-6*, elutants of His-ezrin and only some bound merlin tail.

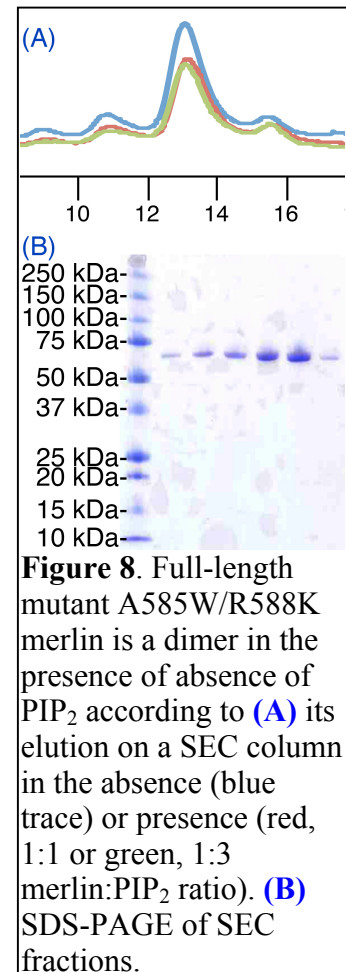


Figure 8. Full-length mutant A585W/R588K merlin is a dimer in the presence or absence of PIP₂ according to (A) its elution on a SEC column in the absence (blue trace) or presence (red, 1:1 or green, 1:3 merlin:PIP₂ ratio). (B) SDS-PAGE of SEC fractions.

3. Key Research Accomplishments

Establishing the expression and purification protocols for full-length human merlin and individual domains will move the neurofibromatosis field forward in fully characterizing the protein responsible for this disease.

4. Reportable Outcomes

Dr. Krishna Chinthalapudi's involvement in this DOD award to generate merlin protein for crystallizations allowed him to obtain a fellowship from the children's tumor foundation to work on other merlin-protein interactions.

Personnel Table

Name:	Dr. Tina Izard
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID:)	0000-0002-2895-483X
Nearest person month worked	All 24 months at about 25% effort
Contribution to Project:	Design and analysis of all experiments
Funding support:	DOD, NIH, and Institutional funds

Name:	Dr. Krishna Chinthalapudi
Project Role:	Post-doctoral fellow
Researcher Identifier (e.g. ORCID ID:)	0000-0003-3669-561X
Nearest person month worked	7 out of 24 months
Contribution to Project:	Design, execution, and analysis of experiments
Funding support:	DOD and NIH

Name:	Dr. Rangarajan Erumbi
Project Role:	Staff Scientist
Researcher Identifier (e.g. ORCID ID:)	0000-0003-3902-8847
Nearest person month worked	14 months at 70% effort
Contribution to Project:	Design, execution, and analysis of experiments
Funding support:	DOD and TSRI Institutional funds

Name:	Dr. Dipak Patil
Project Role:	Post-doctoral fellow
Researcher Identifier (e.g. ORCID ID:)	0000-0003-1112-5557
Nearest person month worked	8 out of 24
Contribution to Project:	Design, execution, and analysis of experiments
Funding support:	Institutional funds

5. Conclusion

Establishing the expression and purification protocols for full-length human merlin and individual domains will move the neurofibromatosis field forward in fully characterizing the protein responsible for this disease. We have made significant progress towards determining the long-sought crystal structure of the human merlin tumor suppressor, and to define how merlin mutations disrupt its structure and function.

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7. Appendices

None

8. Supporting Data

None