Merlin Tumor Suppressor Function is Regulated by PIP_2-Mediated Dimerization.

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Abstract

Neurofibromatosis Type 2 is an inherited disease characterized by Schwann cell tumors of cranial and peripheral nerves. The *NF2* gene encodes Merlin, which contains an N-terminal FERM domain, a central α-helical region and a C-terminal domain that binds to the FERM domain. Changes in the intermolecular FERM-CTD interaction allow Merlin to transition between an open, FERM accessible conformation and a closed, FERM-inaccessible conformation, modulating Merlin activity. These conformational transitions are regulated by both phosphorylation and phosphoinositide binding. Merlin has been shown to dimerize but the role of dimerization in Merlin function is unclear. We used a nanobody based binding assay and found that Merlin dimerizes via a FERM-FERM interaction in a parallel orientation that requires an uncovered N-terminus and the first 18 amino acids of the FERM domain. Patient derived and structural mutants show that dimerization controls interactions with specific binding partners, including HIPPO pathway components, and correlates with tumor suppressor activity. Dimerization requires an open conformation, is inhibited by phosphorylation at serine 518 and is enhanced by PIP_2 binding. The discovery that active, open conformation Merlin is a dimer represents a new paradigm for Merlin function with implications for the development of therapies designed to compensate for Merlin loss.

Introduction

Neurofibromatosis Type 2 is an inherited disease characterized by schwannomas, benign Schwann cell tumors of cranial and peripheral nerves. Sporadic schwannomas with *NF2* mutations represent 8% of all intracranial tumors (1, 2). Targeted deletion of the *Nf2* gene in mouse Schwann cells leads to schwannoma (3, 4) and *Nf2*-null cells have impaired contact inhibition of growth *in vitro* (5, 6). The *NF2* gene encodes Merlin, a 70-kDa member of the Ezrin-Radixin-Moesin (ERM) branch of the band 4.1 superfamily (7, 8). Merlin is predominately localized to the inner face of the plasma membrane (9, 10) where it associates with lipid rafts, membrane microdomains enriched with a variety of signaling molecules (11). Merlin is a component of cell junctional complexes such as adherens junctions and focal adhesions (12, 13). This is consistent with reports indicating that Merlin is a component of the HIPPO pathway, a growth suppressive signaling system responsible for contact inhibition of (14-16). Additionally, Merlin loss activates other oncogenic signaling networks upon Merlin loss, including Ras-ERK, Rac, src, β-catenin, and the mTOR protein kinase complex (6, 17-26). However, the mechanism by which these systems are activated is not defined.

Merlin has a conserved secondary structure consisting of an N-terminal FERM domain followed by a central α-helical (CH) region that folds over itself to form an anti-parallel coiled-coil that positions a C-terminal domain (CTD) for an intramolecular interaction with the FERM domain (5, 27-29). Additionally, Merlin has a unique N-terminal 20 amino acid sequence, absent in other ERM proteins, that is necessary for tumor suppression activity (30). The intramolecular interaction between the Merlin CTD and FERM domains mask a large portion of the FERM domain surface area, resulting in a closed, FERM-inaccessible conformation (31). Upon the release of the CTD from the FERM domain, Merlin transitions to an open conformation that renders the FERM domain accessible to critical interacting proteins (32). A Merlin mutant designed to stabilize the FERM-CTD interaction results in a constitutively closed conformation that has impaired tumor suppressor activity (32). Conversely, an open, FERM-accessible conformation mutant retains activity (32), demonstrating that Merlin's tumor suppressor function is facilitated by the open conformation.

Merlin is phosphorylated at serine 518 by PAK2 (33) and PKA (34). This phosphorylation promotes a closed conformation that down regulates Merlin tumor suppressor activity (32, 35-37). Merlin is also regulated by binding the lipid signaling molecule phosphatidylinositol 4,5 bisphosphate (PIP_2) (38). This interaction promotes Merlin localization to the plasma membrane and is necessary for tumor-suppressive activity (38). PIP_2 binding causes Merlin to assume an open, FERM accessible conformation (39) that is mediated by a structural change in the N-terminal portion of the central α-helical domain that forces the CTD and FERM domains apart (40). These reports show that Merlin activity is determined by conformational changes that are regulated by both phosphorylation and lipid based signaling systems. However, the specific mechanism by which open conformation Merlin interacts with binding partners to facilitate tumor suppression is not known.

The idea that Merlin forms dimers has long been established in the literature, initially by analogy to other ERM proteins and supported by the observation that the central α-helical domain may arrange itself into a coiled-coil structure (41). Experiments using yeast two-hybrid systems (42, 43) and GST pulldowns with isolated Merlin N-terminal FERM and C-terminal domains (44-46)

suggested that Merlin exists as a dimer mediated by intermolecular FERM-CTD binding in an antiparallel orientation. However, experiments using analytical ultracentrifugation explicitly ruled out dimerization (39, 47). Given the contradictory nature of the literature, there is no clear consensus regarding the function of Merlin dimerization and its relationship to conformational regulation. To address these questions, we used a nanobody based assay to measure Merlin binding purified proteins and in co-transfected cell lysates (13). We find that Merlin dimerizes via a FERM-FERM interaction in a parallel orientation that requires the first 18 amino acids of the FERM domain. Patient derived and structural mutants show that the ability to dimerize correlates with tumor suppressor activity. Dimerization requires an open conformation, is inhibited by phosphorylation at serine 518 and is enhanced by PIP_2 binding. These experiments suggest novel mechanistic insights into Merlin function that integrate dimerization with conformation, phosphorylation and phosphatidylinositol signaling,

Results

Merlin Dimerizes via the FERM Domain.

To investigate Merlin dimerization, we adapted a quantitative protein binding assay that utilizes a high affinity anti-GFP nanobody bound to magnetic beads to isolate wild type Merlin-GFP fusion proteins and a probe consisting of Merlin fused to the small, bright luciferase, NanoLuc (Merlin-NL, Figure 1A) (13). The anti-GFP nanobody enabled the purification of Merlin-GFP fusion proteins to homogeneity from transfected HEK 293T cells (Figure 1B). Purified Merlin-NL probes (Figure 1B) were incubated with GFP bound beads, washed, then assayed for luciferase activity. Merlin dimerization was indicated by the amount of luciferase activity normalized to the amount of GFP-Merlin on the beads relative to control (Figure 1C, D). We found that wild-type Merlin-NL bound to Merlin-GFP more than 100-fold greater than control GFP (Figure 1C, D). To map the domains required for interaction we generated deletion mutants of the major Merlin domains, the FERM and CTD (Figure 1E). Normalized binding data from Merlin deletion mutants revealed that the FERM domain is necessary and sufficient for dimerization (Figure 1C, D). The reduced binding to the FH mutant that lacks the CTD and the small amount of binding the HC mutant containing the CTD is evidence of a relatively weak FERM-CTD interaction (Figure 1C, D.). The absence of dimerization in the Merlin-H mutant shows that the central α-helical domain does not interact (Figure 1C, D). This experiment demonstrated that Merlin dimerized via a FERM-FERM interaction.

Merlin Forms a Parallel Dimer

The NanoLuc luciferase substrate emission spectrum peaks 450 nm and is close enough to GFPs excitation peak at 495 nm for bioluminescence resonance energy transfer (BRET), a radiationless biophysical interaction that occurs when NanoLuc and the GFP are less than 10 nm apart (48). Emission at 510 nm from Merlin-GFP:Merlin-NL complexes is evidence of BRET, indicating close proximity of the NanoLuc and the GFP proteins. We took advantage of this property to determine the orientation of the Merlin dimeric complex. HEK 293T cells were co-transfected with Merlin-GFP:Merlin-NL, GFP-Merlin:Merlin-NL, NL-Merlin:Merlin-GFP or GFP-Merlin:NL-Merlin (Figure 1E). Merlin complexes were immunoprecipitated and emission spectra from 400 nm to 600 nm were acquired. There was robust dimerization when GFP and NanoLuc are fused to the Merlin C-terminus (Merlin-GFP:Merlin-NL, Figure 1E). The BRET emission peak at 510 nm is apparent in Merlin-GFP:Merlin-NL, indicating close proximity of the GFP and NanoLuc fusion

proteins in this complex (Figure 1F). When N-terminal fusion and a C-terminal fusion proteins were paired (NL-Merlin:Merlin-GFP or GFP-Merlin:Merlin-NL), significantly less dimerization was apparent (Figure 1E) and the 510 nm peak was much reduced (Figure 1F). When both GFP and NanoLuc are N-terminal, dimerization and the BRET signal was lost (GFP-Merlin:NL-Merlin, Figure 1 E,F). This shows that Merlin dimers exist in a roughly parallel orientation with their C-termini in close proximity. To investigate the stability of the Merlin-Merlin interaction we performed the binding assay at different concentrations of salt. Dimerization was maximal at low salt concentrations (50 mM) and lost at high salt concentrations (300 mM and 600 mM, Figure 1G, H) suggesting that binding is driven predominately by ionic rather than hydrophobic interactions. Taken together, these data confirm that Merlin forms a dimer. Dimer formation requires an uncovered N-terminus and dimers are orientated such that each C-terminus is close to the other, consistent with a parallel orientation.

Merlin Mutant Dimerization

We evaluated the ability of a panel of Merlin mutants to dimerize (Figure 2A). The patient derived mutants Δ39-121, L360P and L535P all showed significantly reduced dimerization relative to wild type (Figure 2B). However, the patient derived L64P mutant showed slightly enhanced dimerization (1.28-fold, Figure 2B). A deletion of the Merlin unique N-terminal 18 amino acids (ΔN18) showed significantly impaired dimerization, and GFP fused to the first 20 N-terminal amino acids (N20) failed to dimerize (Figure 2C) indicating that Merlin's unique N-terminus is necessary but not sufficient for dimerization. A non-phosphorylatable mutant at serine 518, S518A, had significantly enhanced dimerization whereas its phosphomimetic counterpart, S518D, had reduced dimerization (Figure 2C). This suggests that dimerization can be regulated by phosphorylation. Finally, a mutant designed to force Merlin into a constitutively closed conformation, Merlin-AR (32), failed to significantly dimerize relative to wild type. The constitutively open conformation mutant Merlin-ΔEL (32), had significantly increased dimerization (Figure 2C). This showed that dimerization requires Merlin to be in the open conformation. BRET studies confirmed these results, indicating a tighter interaction between S518A dimers than S518D dimers (Figure 2D). The tighter interaction between Merlin ΔEL dimers (Figure 3E), suggests that Merlin dimers are hypophosphorylated and in the open conformation.

Interaction of Dimerization Mutants with Merlin Targets

We hypothesized that Merlin dimerization may affect its interaction with critical target proteins. To test this, we performed co-transfection pulldown experiments with plasmids expressing wild type or mutant Merlin-NLuc and GFP fused the each of four known Merlin binding proteins: Angiomotin, ASPP2, Lats1 and YAP1 (13, 49-51). All four proteins showed significantly reduced binding to the phosphomimetic S518D mutant relative to S518A (Figure 3 A-D). Angiomotin bound to the constitutively closed Merlin-AR mutant but showed impaired binding to the open conformation mutant Merlin-ΔEL (Figure 3A). In contrast, ASPP2, Lats1 and YAP1 all showed either equivalent or significantly increased interaction to Merlin-ΔEL mutant relative to Merlin-AR (Figure 3 B-D). These experiments suggest that dimerization and conformation can affect Merlin's interaction with critical target proteins both positively, for ASPP2, Lats1 and YAP1, and negatively in the case of Angiomotin.

Effect of PIP_2 and Phosphorylation on Dimerization

Structural analyses showed that upon binding to the signaling phosphoinsositide, PIP_2, Merlin assumes an open conformation (39, 40). Since the open conformation correlates with dimerization, we tested if PIP_2 binding also caused enhanced dimerization. First, we tested the lipid binding deficient mutant Merlin-6N and found that it had impaired dimerization (Figure 4A). We then performed binding assays with purified Merlin-GFP and Merlin-NL in increasing concentrations of the soluble PIP_2 analog PIP_2-diC8. This experiment showed increased dimerization from 6.25 to 400 µM PIP_2, with a plateau above 200 µM (Figure 4B). Since the phosphomimetic S518D mutant had impaired dimerization relative to non-phopsphorylatable S518A, we tested if *in vitro* phosphorylation inhibited dimerization in the presence and absence of PIP_2. Merlin dimerization was increased in the presence of PIP_2 by 4.64-fold and phosphorylation by PAK2 decreased the PIP_2 effect significantly (Figure 4C). Western blots confirmed increased levels of phospho-S518 in the PAK2 treated samples (Figure 4C, Inset). These results suggest a regulatory cycle (Figure 4D) in which Merlin is activated by PIP_2, causing it to assume an open conformation that allows dimerization, leading to interactions with target proteins such as ASPP2, Lats1 and YAP1. This "active" tumor suppressing complex is then dismantled upon phosphorylation at S518 by PAK2.

Discussion

We demonstrated that Merlin dimerizes via a FERM-FERM interaction, in a parallel orientation that requires an uncovered N-terminus and the first 18 amino acids of the FERM domain. Patient derived mutants show that the ability to dimerizes correlates with tumor suppressor activity. Dimerization requires an open conformation, is enhanced by PIP_2 binding and inhibited by phosphorylation at serine 518. Experiments conducted with a panel of Merlin deletion mutants demonstrate that the FERM domain is both necessary and sufficient for dimerization. The CTD is not necessary for binding but contributes to the stability of the dimer. Dimerization is significantly reduced in high salt. Given that high salt tends to de-stabilize electrostatic interactions and stabilize hydrophobic interactions within proteins, this rules out dimerization mediated by a coiled-coil structure between adjacent central α-helical domains, suggesting instead that dimerization is mediated by hydrogen bonds and ionic interactions. The FERM-FERM interaction for Merlin has some precedent in the literature. A structure derived from co-crystalized Merlin FERM and CTD domains revealed extensive dyad interactions via the FERM domain, mediated by unfurling the F2 lobe of the FERM domain such that it that interacts with an adjacent FERM from the dimerization partner (52). This structure is consistent with the FERM mediated dimerization that we describe.

Binding studies performed with both N- and C-terminal fusion proteins demonstrate that access to the N-terminus is a necessary Merlin dimerization. This is supported by a study using Merlin with an N-terminal tag that reported it as predominately monomeric (39). This result explains our previous failure to detect Merlin dimers by analytical ultracentrifugation in an intramolecular FRET study of Merlin conformation using Cerulean-Merlin-Venus fusion proteins that are covered at both the N- and C-termini (47). Our results are also consistent with the results from a proximity biotinylation study where N-terminal $BirA^{R118G}$-Merlin labelled substantially fewer Merlin proximal proteins than C-terminal fused Merlin- $BirA^{R118G}$ in living cells (13). This suggests that the ability to dimerize is necessary for Merlin to localize to subcellular target structures like focal adhesions and adherens junctions. The Merlin-Δ18 and N20 mutants show that the extreme N-terminus is necessary but not sufficient for interaction. This region is required for Merlin mediated growth suppression and localizes Merlin to the inner

face of the plasma membrane (30) where it is a target of regulatory kinases that control Merlin stability (37, 53).

The behavior of the conformation mutants, Merlin-AR and Merlin-ΔEL, show that the open conformation is necessary for dimerization. The inability of Merlin-AR to dimerize is telling. Merlin-AR is an inactivating mutant that has amino acid substitutions in the extreme C-terminus, A585W and R588K, that stabilize the intramolecular FERM-CTD interaction (32) thus fixing Merlin into the "closed' conformation. In contrast Merlin-ΔEL has a deletion of the last two C-terminal amino acids, E594 and L595, that weakens the intramolecular FERM-CTD interaction leading to a more "open" conformation (32). This mutant retains tumor suppressor activity (32) and has enhanced dimerization relative to wild type, again linking dimerization with the open conformation and tumor suppressor function. Also, Merlin-ΔEL shows enhanced binding to three of four Merlin binding proteins, ASPP2, YAP1 and Lats1. This result suggests that dimeric Merlin complexes are responsible for binding to critical target proteins that mediate tumor suppressor function. In contrast, the interaction of Angiomotin to Merlin-ΔEL is reduced relative to Merlin AR and wild type, suggesting that the Angiomotin favors the closed conformation and perhaps has a function that is distinct from the other Merlin binding proteins.

Dimerization is impaired in the phosphomimetic mutant S518D and by phosphorylation of S518 by PAK2 *in vitro*. Furthermore, binding of S518D with Angiomotin, ASPP2, YAP1 and Lats1 is reduced relative to both S518A and wild type. These results support the idea that phosphorylation at serine 518 inactivates Merlin tumor suppressor activity by disassociating dimeric complexes. The inability of the lipid binding mutant Merlin-6N to dimerize implicates phosphoinositol signaling as a means of regulating dimerization. The significant increase in dimerization with increasing concentrations of PIP_2 confirms that dimerization is positively regulated by PIP_2 binding. This result is consistent with structural studies showing that PIP_2 binding causes conformational changes in the central α-helical domain that result in an active, open conformation (40). The reduction of PIP_2 mediated dimerization after phosphorylation at S518 by PAK2 is supported by a small angle neutron scattering study demonstrating that, unlike wild type, the S518D mutants fail to adopt an open conformation upon PIP_2 binding (39).

Together these results suggest a regulatory loop in which hypo-phosphorylated Merlin is activated by PIP_2 causing a shift to the open conformation followed by dimerization and interaction with binding partners. This active complex is then phosphorylated on serine 518 by PAK2 leading to a closed, inactive conformation (Figure 4D). Merlin is often described in the literature as an adaptor molecule that acts to target specific proteins to the inner face of the plasma membrane. The idea that Merlin dimers represent active complexes is consistent with these functions. It is possible each partner of a Merlin dimer may bind to a different target and thus facilitate interaction between them, as has been shown for Lats1 and YAP1 (50). The model we propose for Merlin function may also be relevant therapeutically. One strategy to treat NF2 proposes to use gene therapy to replace lost Merlin function in schwannomas. The ability of Merlin to dimerize raises the possibility that some mutant Merlin proteins may act as dominant negative mutants that impair the activity of ectopic wild type Merlin, lessening its therapeutic effect. Understanding the biochemical mechanism of Merlin function will give insight into the consequences of overexpressing Merlin in this context and is therefore critical to the overall effectiveness of these strategies.

Materials and Methods

pEGFP C3-hYAP1 and pEGFP C3-Lats1 (Addgene plasmid nos. 19053 and 17843, respectively) were gifts from M. Sudol. Construction of pGFP-AMOT, pMer-GFPst and pMer-NLst were described previously (13). Merlin mutants were introduced into pMer-GFPst and pMer-NLst by amplification of the entire plasmid using mutant or deletion specific primers (IDT, Coralville, IA) with Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA). Amplified plasmids were circularized using NEBuilder HiFi DNA Assembly Cloning Kit then transformed into NEB-α Competent Cells (New England Biolabs, Ipswich, MA). All cloning was confirmed by sequencing.

Cell Culture

HEK-293T cells were grown in DMEM supplemented with 10% FBS and Pen/Strep at 37ºC 5% CO_2.

Protein Purification

HEK 293T cells (5 x 10^6) were plated into each of two 15-cm dishes and then transfected with 50 µg of pMerlin-NLuc-StrepTag plasmid per plate using polyethylenimine (PEI, Sigma Aldrich, St Louis MO). Cells were harvested and Merlin-NL was purified as previously described. (13). GFP-bait expressing plasmid was transfected into 2 x 10^6 HEK 293T cells in a 10 cm dish, lysed as described above then affinity purified with GFP-Trap_MA beads as per manufacturers instructions (Bulldog Bio,.

Direct Binding Assays

The GFP bound beads were resuspended in a 30-µl blocking buffer of 0.5 mg/ml BSA in TBST and incubated for 1 hour at room temp., The beads were recovered magnetically then resuspended blocking buffer plus 30 to 50 nM Merlin-NLuc protein and incubated at room temperature for 1 hour. Beads were then recovered, washed four times with TBST the resuspended in 25 µl TBST. NLuc luciferase activity was measured using NanoGlo Luciferase Substrate Buffer (Promega, Madison, WI). NanoLuc activity, GFP fluorescence and BRET spectra were measured on a FlexStation 3 (Molecular Devices, San Jose, CA).

Co-Immunoprecipitation.

HEK 293T cells were co-transfected with GFP-target and Merlin- NLuc plasmids, lysed in 0.5 ml of TBS, 2 mM $MgCl_2$, 0.5% NP-40, protease inhibitor mix. Lysates were diluted 1:2.5 with TBST then NLuc activity was measured. GFP fusion proteins were immunoprecipitated using GFP-Trap_MA (ChromoTek, Hauppauge, NY), recovered using a magnetic stand, and washed four times with TBST and NLuc luciferase activity was measured as described above.

PIP_2 and PAK2.

Active PAK2 (Sigma-Aldrich) was incubated with purified Merlin-GFP bound beads and Merlin-Nluc in 25 mM Tris (pH 7.4), 2 mM DTT, 10 mM $MgCl_2$, 200 µM ATP, 150 mM $NaCl_2$, +/- 200 µM PiP_2-diC8 (Eschelon Biosciences), +/- 0.278 U/µl PAK2 for 1 hour at 30ºC. Nluc activity and BRET was measured as described above.

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Figures

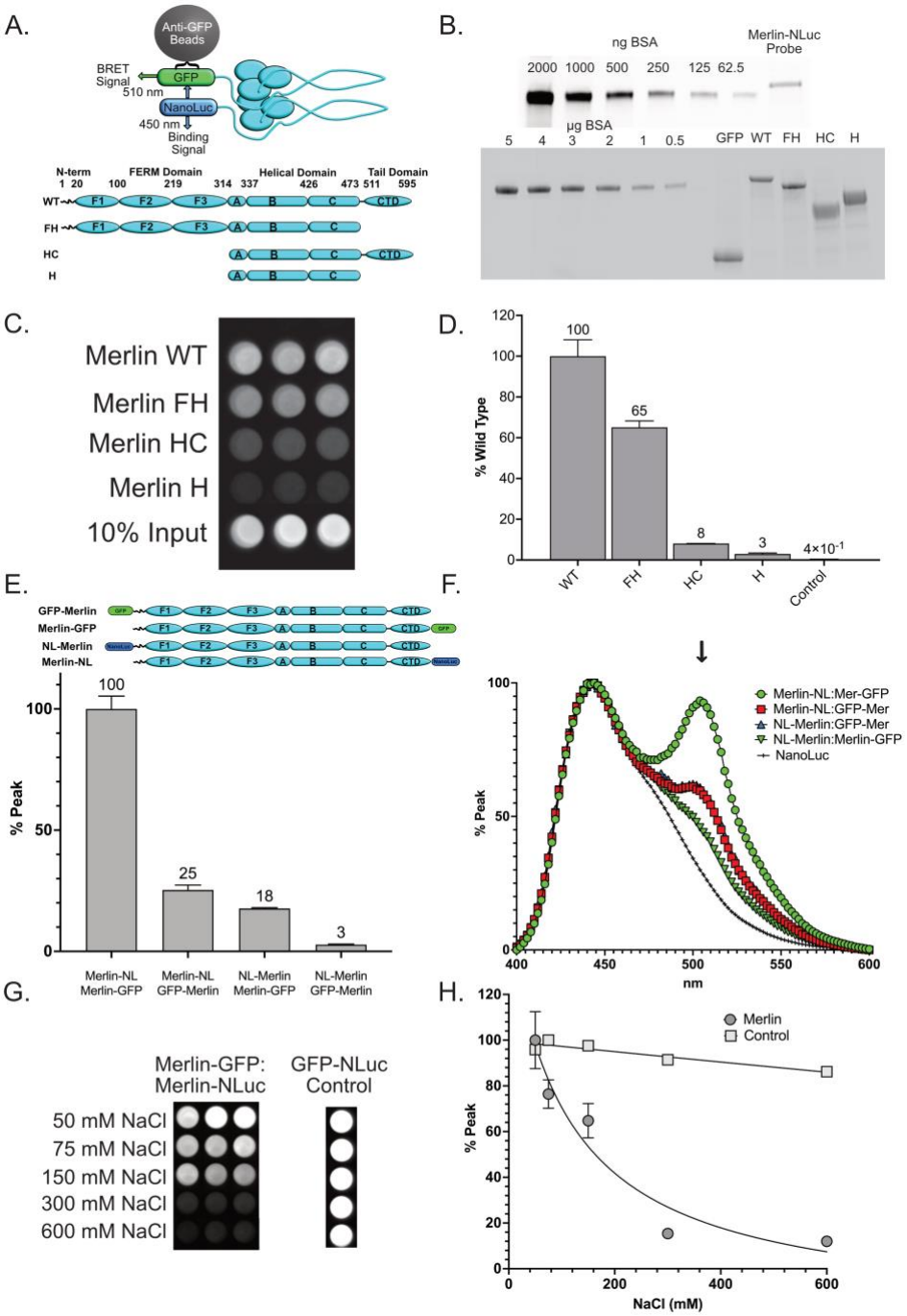

Figure 1.

A. A schematic diagram depicting the dimerization assay and major domain mutants for Merlin. B. SDS-PAGE gels stained for total protein to access the quantity and purity of isolated NanoLuc probes for Merlin-NL (top) and GFP "bait" proteins (bottom) for GFP, Merlin-GFP, Merlin-FH-GFP, Merlin-HC-GFP and Merlin-H-GFP. C. An image of NanoLuc activity in binding assays

between Merlin-WT (left) and Merlin-FH and the GFP- “Bait” protein GFP (control), WT, FH, HC and H. D. Normalized binding data from C. E. Triplicate Merlin dimerization assays for the N- and C-terminal Merlin constructs, normalized to GFP fluorescence. Inset: A diagram showing the Merlin binding/BRET assay and a schematic diagram depicting the Merlin domain structure and the N- and C-terminal GFP fused “bait” and the N- and C-terminal fused “probe“constructs. F. Emission spectrum from 400 nm to 600 nm of Merlin dimerization assays normalized to 450 nm peak. The 510 nm BRET emission peak is indicated by the arrow. G. Dimerization assays performed at 50 mM, 75 mM, 150 mM, 300 mM and 600 mM NaCl. The GFP-NanoLuc fusion protein used to control for the effects of salt on luminescence. H. Graphical representation of normalized dimerization with salt titration.

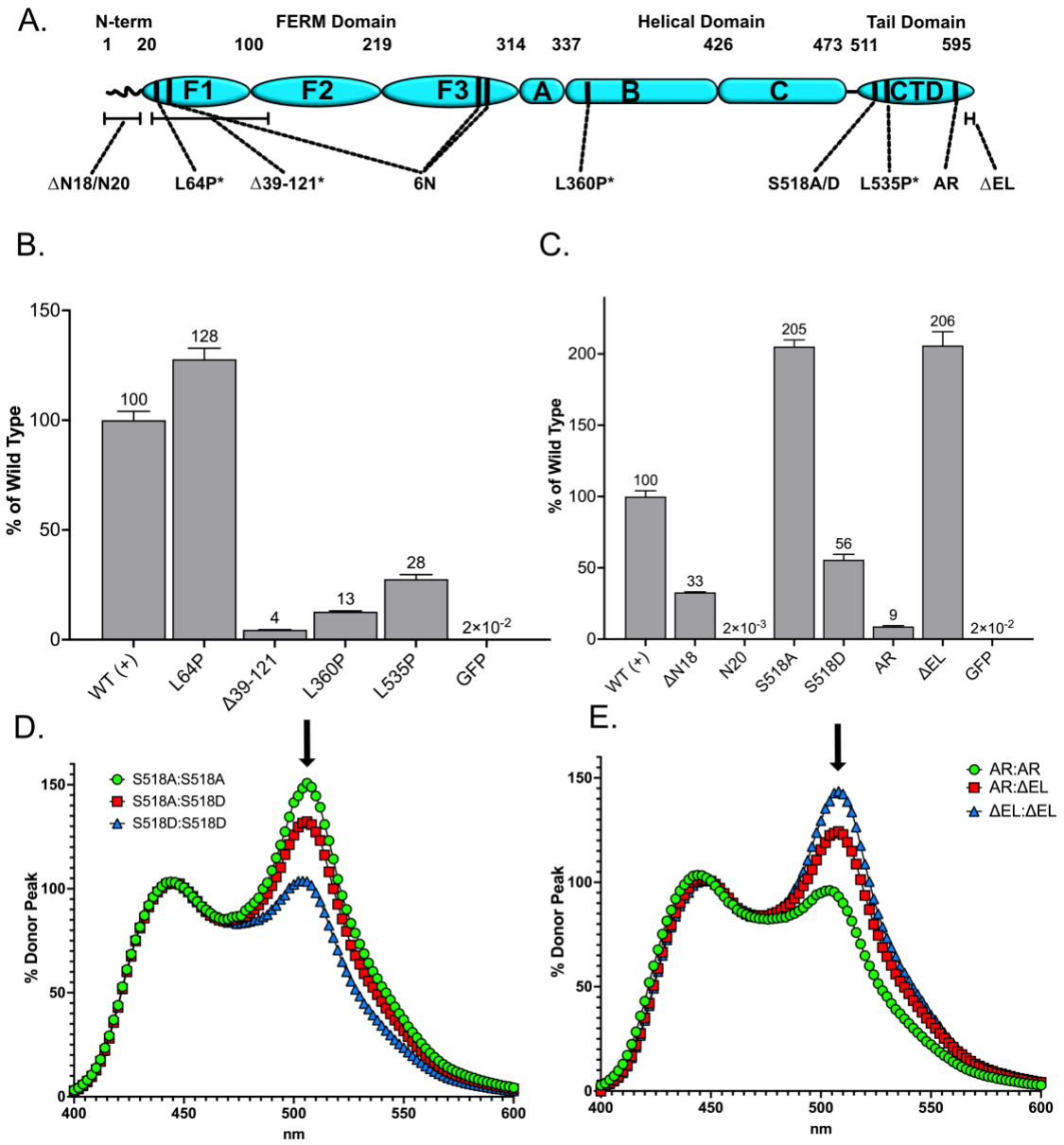

Figure 2. A. A schematic diagram of Merlin mutants. Patient derived mutations: L64P, L360P and L535P and indicated by asterisks. ΔN18: deletion of the N-terminal 18 amino acids. N20: the first 20 amino acids fused to GFP. S518A/S518D: non-phosphoylatable and phosphomemetic mutants at S518. AR: closed conformation mutant. ΔEL: open conformation mutant. B. Pulldown dimerization assays of patient derived Merlin mutants. C. Pulldown dimerization assays of Merlin N-terminal, phosphorylation and conformation mutants. D. BRET emission spectrum from 400 nm to 600 nm of phosphorylation mutant dimer pairs normalized to 450 nm, 510 nm BRET peak indicated by the arrows. E. BRET assays for conformation mutant dimer pairs.

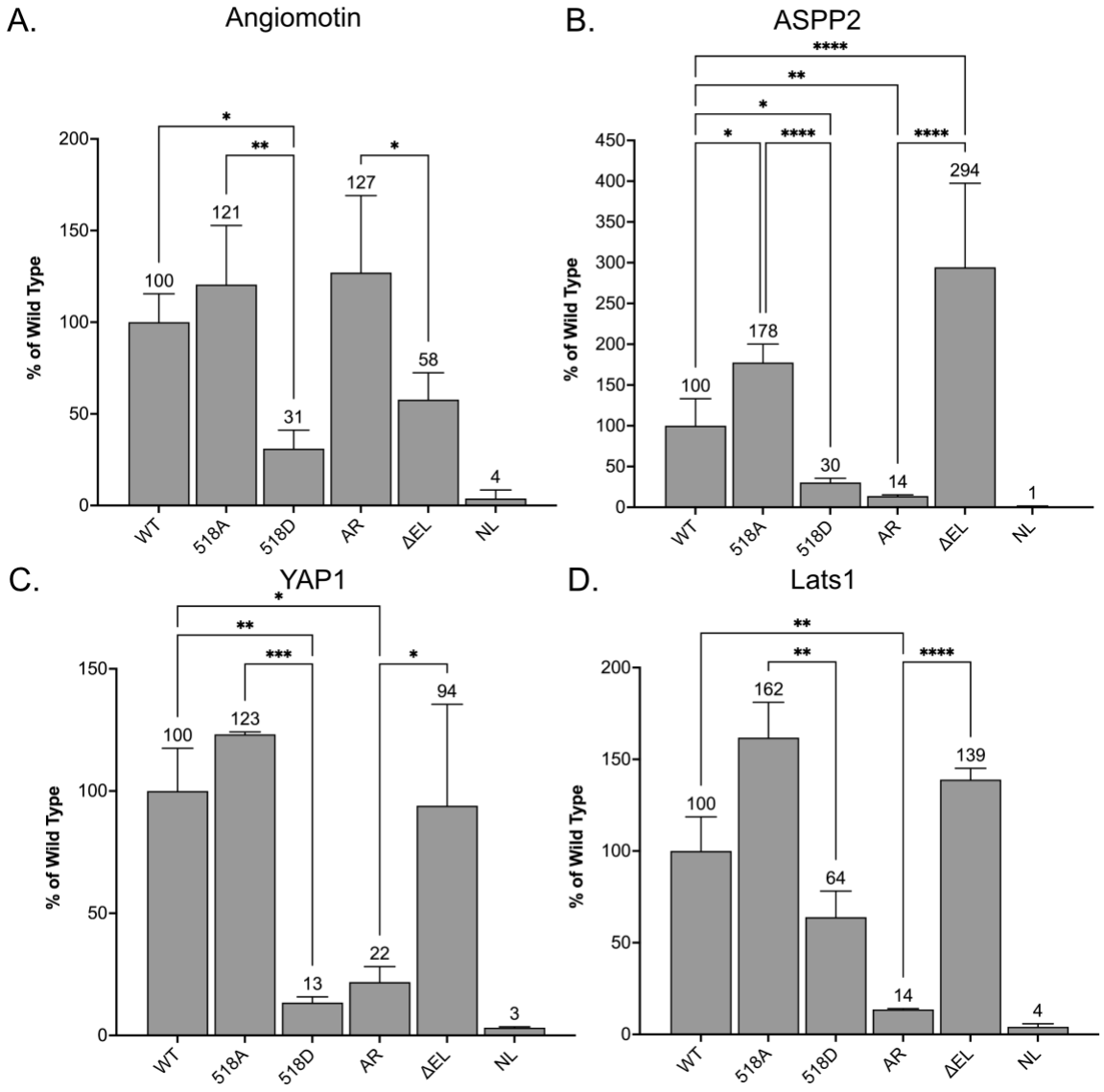

Figure 3. Normalized NanoLuc activity of GFP pulldowns from cells transfected with plasmids expressing wild type and mutant Merlin-NLuc and GFP fusions for angiomotin (A.) ASPP2 (B.), Lats1 (C.) and YAP1 (D.) Significant differences are indicated by the bars and asterisks.

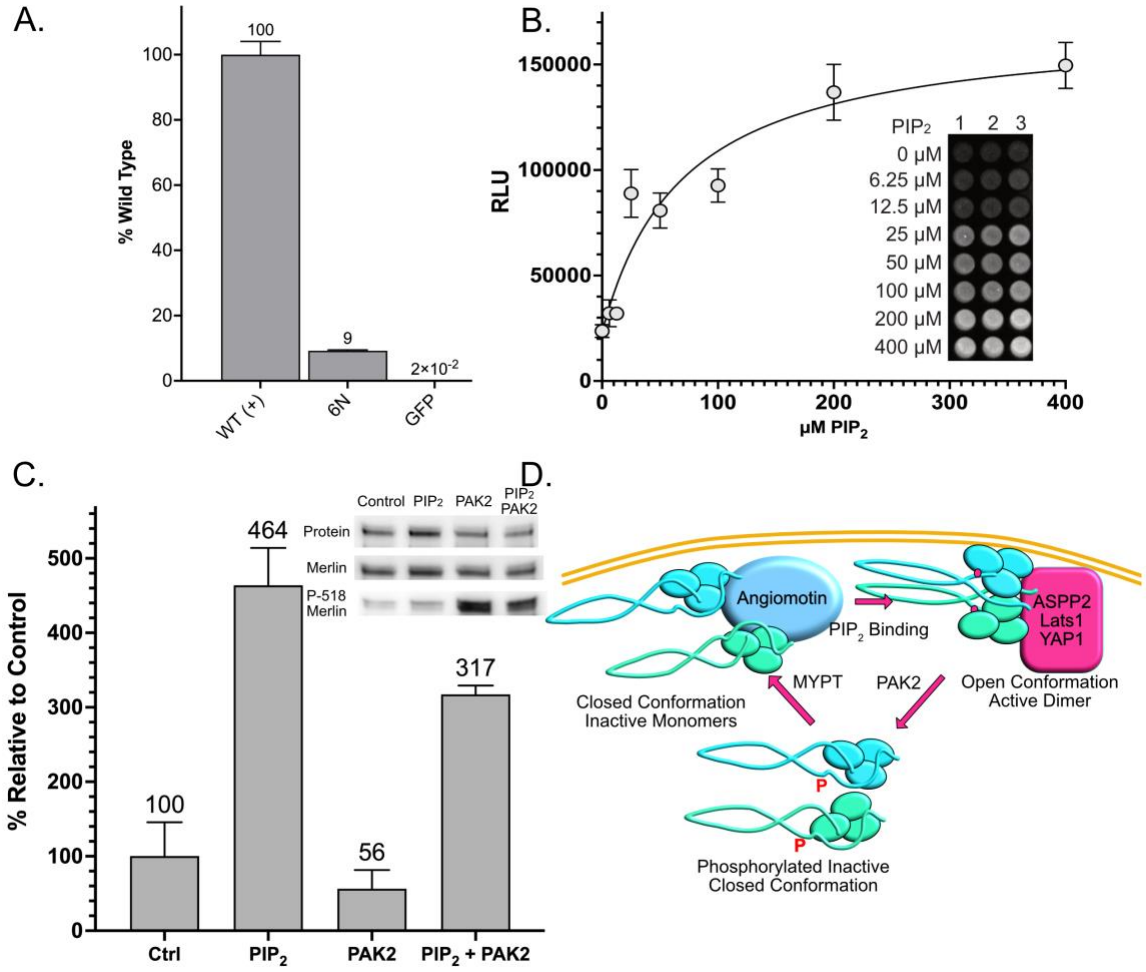

Figure 4. A. Dimerization assay for the Merlin-6N lipid binding deficient mutant expressed relative to wild type. B. Dimerization assay using purified Merlin-GFP and Merlin-NLuc incubated with increasing concentrations of PIP_2-DiC8. Inset, an image of the PIP_2 titration microtiter plate. C. Emission spectrum of Merlin-GFP:Merlin-NLuc dimers in the presence and absence of PIP_2. D. Model for Merlin activation cycle. Inactive closed conformation associated with Angiomotin. PIP_2 binding leading to open conformation, dimerization and binding to ASPP2/Lats1/YAP1. Active complexes are phosphorylated leading to dissolution of the complex followed by dephosphorylation to regenerate the Merlin-Angiomotin complex.