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The conformation change and tumor suppressor role of Merlin are both independent of Serine 518 phosphorylation

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ABSTRACT

Merlin functions as a tumor suppressor and suppresses malignant activity of cancer cells through multiple mechanisms. However, whether Serine 518 phosphorylation regulates the conformation of Merlin as well as the open-closed conformational changes affect Merlin's tumor inhibitory activity remain controversial. In this study, we used different mutants to mimic related conformational states of Merlin and investigated its physiological functions. Our results showed that the phosphorylation at Serine 518 has no influence on Merlin's conformation, subcellular localization, or cell proliferation inhibitory activity. As a fully closed conformational state, the A585W mutant loses the ability to recruit Lats2 to the cell membrane, but it does not affect its subcellular distribution or cell proliferation inhibitory activity. As a fully open conformational state, mimicking the conformation of Merlin isoform II, the ΔEL mutant has the same physiological function as the wild type Merlin isoform I. Collectively, we provide for the first time in *vivo* evidence that the function of Merlin, as a tumor suppressor is independent of its conformational change.

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1. Introduction

Merlin was discovered as a tumor suppressor that functions in the neurofibromatosis type 2 (NF2) more than two decades ago [1,2]. NF2 patients develop meningioma and schwannoma. In the general population, loss of NF2 correlates with the other cancer types including mesothelioma, glioma multiforme, colorectal, breast, skin, hepatic and prostate cancer [3]. Merlin is similar to some members of ERM (ezrin, radixin and moesin) family of proteins that are composed of three distinct domains: N-terminal domain (referred to as FERM domain), central coiled-coil domain and C-terminal domain (referred to as CTD domain) [4] and are thought to link the cytoskeleton to cell membrane proteins [5,6].

Merlin has two different isoforms *in vivo*: isoform I and isoform II. Isoform I is a longer predominant one and isoform II only lacks five amino acids at the C-terminus compared to isoform I [7,8]. Merlin isoform I possesses a relatively "closed" conformation via a FERM/CTD intramolecular interaction. Evidence from a few studies suggests that Merlin isoform I can be phosphorylated on Serine 518 (referred to as Ser518) by kinases of the P21-activated kinase (PAK) family or Protein Kinase-A (PKA) [9–11]. Previous studies also suggest that the phosphorylation of Ser518 can convert Merlin isoform I into an inactive state in suppressing cell growth [12–14]. In contrast, the alternatively spliced Merlin isoform II exists in a relatively "open" conformation [15,16]. Merlin isoform II has been shown unable to function in contact inhibition or tumor suppression [17], which seems to function similarly as Merlin isoform I in an open conformation. However, three other independent groups found that Merlin isoform II suppresses growth in mammalian cell lines [18–20], suggesting that interdomain binding may not be necessary for Merlin's growth inhibitory activity. Moreover, FRET (fluorescence resonance energy transfer) studies revealed that Merlin's FERM domain and CTD domain are constitutively held in close proximity, undergoing only subtle changes upon post-translational modification [21]. Overall, it remains controversial whether the phosphorylation modification at Ser518 regulates the open-closed conformational change of Merlin and thus its growth inhibitory activity.

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Our previous study showed that residue Ser518 is located outside Merlin's auto-inhibitory tail and its phosphorylation does not dramatically alter Merlin's conformation, but instead prevents Angiomotin from binding and thus inhibits Hippo pathway activation [4]. Prior data indicated that a mutant missing the last two amino acid residues (referred to as ΔEL) is in an open conformation and may mimic the conformation of Merlin isoform II, which has a shorter C-terminus and could bind EBP50 effectively [18,22]. As a strongly closed conformation control, we substituted Alanine 585 of Merlin with Tryptophane (referred to as A585W) based on sequence alignment of Merlin CTD and EBP50, and subsequently mutant A585W loses the ability of binding to EBP50 [4,18].

However, whether and how the phosphorylation of Serine 518 and the open or closed conformation of Merlin affect cell growth inhibition in *vivo* remain to be further revealed. Here, we used these mutants to study Merlin's subcellular localization, its ability to recruit Lats2 to the cell membrane and its tumor suppressor role. Our results may shed light on the physiological functions of Merlin and the treatment for Merlin-deficient tumors.

2. Materials and methods

2.1. Gene cloning and protein purification

The coding sequence of *Merlin* was cloned into the pEGFP-C1 vector and the home-modified pET-32a vector (Novagen, USA) in which the Trx-tag was deleted and the S-tag and thrombin recognition sites were replaced by PreScission protease cleavable segments. The coding sequence of *Lats2* was cloned into pCMV-Myc and pmCherry-C1 vectors. All Merlin mutants (S518A, S518D, A585W and ΔEL) were created using a standard PCR-based mutagenesis method and confirmed by DNA sequencing. The recombinant protein was expressed in BL21 (DE3) *Escherichia coli* cells at 16 °C for 16–18 h. All His_6-tagged Merlin proteins were purified by HisTrap HP column (GE Healthcare, USA) followed by size exclusion chromatography on a Superdex 200 10/300 GL column (GE Healthcare, USA) in Tris-HCl buffer (50 mM Tris pH7.5, 150 mM NaCl and 1 mM DTT).

2.2. Circular dichroism

Circular dichroism (CD) spectroscopy of Merlin was performed on a MOS450 spectropolarimeter (BioLogic, USA) at room temperature. The protein samples (6 μM) were dissolved in Tris-HCl buffer (50 mM Tris pH7.5, 150 mM NaCl and 1 mM DTT). As the temperature increases from 25 °C to 90 °C, the absorption at wavelength of 220 nm was detected.

2.3. Lipids binding assay

Brain lipid extracts (Sigma, USA) were resuspended at 2 mg/ml in a buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, and 1 mM DTT. The protein samples (5 μg) were incubated with liposomes (30 μg) for 15 min at room temperature in a 40 μL volume. After the binding reaction, all the samples were centrifuged at 100,000 rpm for 40 min at 4 °C in Beckman MAX XP. After centrifuge, supernatant that was not bound to liposomes was carefully removed. The pellet was washed twice with the same buffer and brought up to the same volume as the supernatant which had been removed. Finally, 10 μL each of supernatant and pellet proteins was loaded onto SDS-PAGE gels and visualized by Coomassie blue staining.

2.4. Cell culture and transfection

Hela, HEK293T and MDA-MB-231 cells were grown in Dulbecco's Modified Eagle's Medium (Gibco, USA), supplemented with 10% fetal bovine serum (Gibco, USA) at 37 °C with 5% CO_2 atmosphere in a humidified incubator. All transfections were performed using polyetherimide (Polysciences, USA) according to the manufacturer's protocol.

2.5. Immunofluorescence and confocal

Hela cells were seeded to a 24-well plate with a coverslip in each well. After reaching appropriate density, the cells were washed twice in PBS and then fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. After being rinsed three times with PBS, the cells were counterstained with DAPI and mounted onto slides. The images were acquired using Leica TCS SP5 laser scanning confocal microscope with a 40× oil immersion objective (Leica Microsystems, Germany) and analyzed by Photoshop.

2.6. Co-immunoprecipitation and western blotting

HEK293T cells were co-transfected with the plasmids containing Merlin and Lats2. After 24 h of transfection, the cells were lysed by ice-cold lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP40, 10% glycerol, and protease inhibitor cocktail). The lysate was clarified by centrifuge at 13,000 rpm for 15 min at 4 °C. 30 μL of cell lysate was saved as input, and the rest was incubated with beads conjugated with GFP antibody for1 h at 4 °C. Afterwards, the beads were washed with lysis buffer three times and the samples were eluted with 2× loading buffer. All samples were separated by SDS-PAGE gels and transferred to PVDF membrane (GE Healthcare, USA). The PVDF membrane was immunoblotted with anti-Myc (Sigma, USA) or anti-GFP (Sigma, USA) antibodies, and was then probed with horseradish peroxidase conjugated secondary antibodies (Santa Cruz, USA). The images were acquired using a chemiluminescent substrate (Millipore, USA) by Tanon 5200 Multi (Tanon, China).

2.7. Cell proliferation assay

MDA-MB-231 cells were transfected with wild-type or mutant (S518A, S518D, A585W and ΔEL) Merlin expressing plasmids by polyetherimide. 48 h after transfection, the cells were counted by a blood counting chamber and 1000 cells/well were seeded in 96-well plates in quintuplicate. Cells were incubated at 37 °C in a humidified incubator with 5% CO_2 and allowed to grow up to 5 days. Cell proliferation assays were performed using cell counting kit-8 (referred to as CCK-8, Beyotime, China) following the manufacturer's instruction. Experiments were repeated three times, and data were represented as the mean ± SEM.

2.8. Plate colony formation assay

MDA-MB-231 cells were transfected with wild-type or mutant (S518A, S518D, A585W and ΔEL) Merlin expressing plasmids by polyetherimide. 48 h after transfection, 500 cells/well were seeded in a 6-well plate. After cultured for 14 days, all the colonies were washed with PBS twice, fixed with formaldehyde for 20 min at room temperature, and then stained with 0.1% crystal violet. Finally, the image is scanned after washing with PBS three times.

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