The promotion of neurite formation in Neuro2A cells by mouse Mob2 protein

Cheng-Han Lin, Mingli Hsieh, Seng-Sheen Fan*

Department of Life Science, Tunghai University, Taiwan, Republic of China

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ABSTRACT

The molecular mechanism of neuritogenesis has been extensively studied but remains unclear. In this study, we identified Mob2 protein which plays a significant role in promoting neurite formation in Neuro2A (N2A) cells. Our results showed that Mob2 was expressed in developing N2A cells. To study whether Mob2 was involved in neurite formation, we downregulated Mob2 expression using RNA interference and found that neurite formation decreased in low serum induced N2A cells. In addition, we found that overexpression of Mob2 promoted neurite formation in N2A cells. Furthermore, downregulation of Mob2 expression altered the rearrangement of the actin cytoskeleton and decreased the expression of phosphorylated Moesin. Together, these results provide information on the role of Mob2 in mediating neurite formation.

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1. Introduction

Mps-one-binder (Mob) family proteins belong to a conserved gene family found in yeast [1–3], Drosophila [4,5], and mammals [6]. Mob1 protein is originally identified as a regulator of mitotic exit and cytokinesis in yeast [1]. In Drosophila, Mob1 acts as a tumor suppressor by its functions as a key component of the Hippo signaling pathway [5,7]. Mob2 binds to Cbk1, a conserved protein kinase, which is similar to the human myotonic dystrophy kinase. Mob2 promotes polarized cell growth and induces asymmetric cell fate in fission yeast [3,8–10]. In Drosophila, the Mob2 protein physically binds to Tricornered protein, an orthologue of NDR kinase [11]. Study also shows that Dmob2 involves in photoreceptor morphogenesis by regulating the subcellular localization of Crumbs, a cell polarity gene, and phosphorylated Moesin [12]. Knocking down Dmob2 expression causes abnormal actin rearrangement and results in abnormal rhabdomere formation, which suggests that Dmob2 may regulate actin dynamics during rhabdomere formation [12].

The nuclear Dbf2-related (Ndr) kinase belongs to a subfamily of serine/threonine protein kinases that control cell division and morphogenesis in various cell types [13]. Ndr2, one of the Ndr kinases, is induced in the mouse amygdale during fear memory consolidation. Study further demonstrates this kinase plays an important role in neurite formation [14]. The Ndr kinase orthologue in Drosophila, tricornered interacts with actin filament and regulates dendritic tiling and branching [11,15]. SAX-1, the orthologue of Ndr kinase in nematodes, activates the RhoA-GTPase signaling pathway during neurite formation [16]. These results strongly indicate the importance of Ndr kinases in neurite formation. Mob proteins have been shown to bind Ndr kinases, making them important in kinase activation [6,17–19]. In this study, we investigated what role, if any, Mob proteins play in neuritogenesis.

In this study, we investigated whether the Mob2 protein in mice plays a similar role in organizing actin filaments and regulating neurite formation during development. We used neuro 2A cells (N2A), a mouse neuroblastoma cell line, to study how Mob2 participates in neuritogenesis. Immunocytochemical study indicated that Mob2 localization in cytoplasm and on the tip of neurite protrusion. Using RNA interference to knockdown Mob2 expression and found that neurite formation was suppressed in low-serum induced N2A cells whereas overexpression of Mob2 induced neurite formation in normal serum growth N2A cells. Taken together, our results provide evidence that demonstrate the function of Mob2 in neurite formation.

2. Materials and methods

2.1. Molecular biology

RT-PCR was performed on a mouse brain to construct expression vectors encoded by mob1A, mob1B, and mob2. These genes were cloned onto the pcDNA™ 3.1/myc-His.B expression vector to generate pcDNA-mob1A, pcDNA-mob1B, and pcDNA-mob2 (Invitrogen Life Tech., Carlsbad, CA). To construct expression vectors encoded by ndr2, RT-PCR was performed on a mouse brain. The gene was cloned onto the pAGFP expression vector to generate pAGFP-ndr2 (Clontech, CA). To generate the anti-Mob2 antibody, the full-length mob2 PCR product was cloned to the pGE-4T-1 vector (GE

* Corresponding author. Address: Department of Life Science, Tunghai University, No. 181, Sec. 3, Taichung-Port Road, Taichung, 407 Taiwan, Republic of China. Fax: +886 4 23590296.

E-mail address: sfan@thu.edu.tw (S.-S. Fan).
Healthcare) to make pGEX-4T-mob2. All constructs were verified by DNA sequencing before processing for experiments.

2.2. Mob2 RNA interference

To knockdown the endogenous Mob2 expression, the pLKO.1 short hairpin RNA (shRNA) plasmids encoding shRNA with scrambled sequences (Clone ID: TRCN0000072179) or sequences targeting mouse mob2 (mob2-shRNA 1, Clone ID: TRCN0000125837, corresponding coding sequence: GCAGTATAGCACAATCTCAGA; mob2-shRNA 2, Clone ID: TRCN0000125836, corresponding coding sequence: GCTAAGCCAAACGGCAAGAAA) were purchased from the National RNAi Core Facility, Taiwan.

2.3. Culture and transfection of N2A cells

Neuro2A cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM l-Glutamine, 1% PS (penicillin/streptomycin), and 1% non-essential amino acids maintained at 37°C in a humidified atmosphere with 5% CO2. Lipofectamine™ and PLUS™ reagents (Invitrogen Life Tech., Carlsbad, CA) were used for the transfection. For shRNA transfection, 3 μg/ml of puromycin was added to select the transfected cells. After the transfection, the N2A cells were collected and processed for immunofluorescence and Western blotting.

2.4. Antibody production

To generate the anti-Mob2 antibody, the pGEX-4T-mob2 plasmids were transformed into E. coli (BL21). After IPTG induction, the cell lysate were harvested and separated with SDS–PAGE. The 56 kDa GST-Mob2 recombinant protein were excised from gel, mixed with adjuvant, and injected into rabbits. After several boosts, the serum was collected and tested for immunoreactivity using Western blot and immunocytochemistry.

Fig. 1. Subcellular localization of Mob2 in differentiating N2A cells. Confocal images showed low-serum induced N2A cells stained with anti-Mob2 (green), rhodamine–phalloidin (red), and anti-tubulin (blue). (A) In differentiatated N2A cells with short neurites, the Mob2 expression was mainly in the cytoplasm and at the base of the protrusive neurite (arrows). (B) In differentiated N2A cells with long neurites, Mob2 expression was concentrated at the base of protrusive neurite, the branching point of the neurites, and the tip of the neurites. (C) Higher magnification of B showed that Mob2 stained the base of protrusive neurite (arrow), the branching point of the neurites (asterisk), and at the tips of the neurite (arrowhead). (D) Higher magnification of B showed that Mob2 stained the bulge-like structures in a neurite (arrowheads). Scale bars: 20 μm.
2.5. Immunofluorescence

For immunofluorescence, the cells were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) for 20 min. After three washes and blocking with 5% FBS, the cells were incubated with primary antibody, rhodamine–phalloidin for F-actin (Sigma–Aldrich, St. Louis, MO), or SYTOX® Green for nucleus (Invitrogen Life Tech., Carlsbad, CA) at 4 °C overnight or at 20 min. After three washes and blocking with 5% FBS, the cells were incubated with primary antibody, rhodamine–phalloidin for F-actin (Sigma–Aldrich, St. Louis, MO), or SYTOX® Green for nucleus (Invitrogen Life Tech., Carlsbad, CA) at 4 °C overnight or at
RT for 1.5 h. The primary antibodies used in this study included anti-tubulin (Sigma–Aldrich, St. Louis, MO), anti-p-Moesin (Santa Cruz Biotechnology) and anti-cMyc (Santa Cruz Biotechnology). After washes and mounting, the cells were examined on a Zeiss LSM 510 confocal microscope. Images were processed using Adobe Photoshop 7.0 software.

2.6. Quantification of neurite outgrowth

The qualification of neurite growth was based on a previous study [20]. Primary cell with neurite formation is defined as an outgrowth from the cell body with a length longer than half the diameter of the cell body. At least 300 cells per treatment were scored. The data presented in this study consisted of the mean of three independent experiments. The results were compared by using the Student’s t-test.

3. Results

3.1. Mob2 expression during neuritogenesis

To detect Mob2 expression in neuronal cells, anti-Mob2 antibody was generated (Fig. S1). Western blot analysis indicated that Mob2 expression increased significantly at 24 h and remained steady until at least 72 h after low-serum induction (three experiments, Student’s t-test, **P < 0.001) (Fig. S2). Immunocytochemistry demonstrated that the Mob2 protein was mainly localized at the cytoplasm and the cellular protrusion in differentiated N2A cells with short neurites (Fig. 1A). In differentiated N2A cells with long neurites (Fig. 1B and D), Mob2 was localized at the base of the neurite (Fig. 1C, arrow), the branching points of the neurite (Fig. 1C, asterisk), and at the tips of the neurite (Fig. 1C, arrowhead). In addition, we often found Mob2 stained the bulge-like structure in a long neurite (Fig. 1D, arrowheads). Together, these results demonstrate that subcellular localization of Mob2 is similar to the position of neurite protrusion suggesting a potential function for the Mob2 protein in neurite formation.

3.2. Downregulation of Mob2 prevents neurite outgrowth

To study the function of Mob2 in neurite formation, RNA interference was used to downregulate Mob2 expression. N2A cells were transfected with two distinct shRNAs that targeted the mob2 sequence and control scramble shRNAs, individually. After puromycin selection and low-serum induction, the level of Mob2 expression in N2A cells decreased to 72% and 73%, respectively, when compared to control shRNA (three experiments, Student’s t-test, **P < 0.001) (Fig. 2A). To study whether downregulation of Mob2 affects neuritogenesis, N2A cells were individually transfected with mob2 and control shRNAs. The transfected cells were stained with anti-Mob2, rhodamine–phalloidin, and sytox to assay for their ability of neurite formation. When N2A cells were treated with control shRNA, the cells expressed Mob2 and extended neurites normally as the untransfected cells (Fig. 2B). When cells were treated with mob2-shRNAs, the expression of the Mob2 protein was significantly reduced in the N2A cells (Fig. 2B). Those cells lacking Mob2 expression usually had short or no neurites (Fig. 2B).

To determine the specificity of mob2-shRNAs, N2A cells were cotransfected with mob2-shRNAs and a mutant version of mob2 gene, which it expresses a protein same as wild type Mob2 protein but do make the mRNA resistant to shRNA (Fig. 2C). If the phenotype observed in N2A cells was mob2 specific, we were then able to observe that expression of the mutant version of mob2 gene should rescue the mob2-shRNAs induced phenotype. Indeed, the results indicated that the simultaneous expression of mob2-shRNA and mutant version of mob2 were able to induce neuritogenesis (Fig. 2D, arrows). Quantitative analysis showed that 75.5 ± 8.5% of cells bore neurites in the control shRNA while the percentage of cells bearing neurites in mob2-shRNA treated cells was significantly reduced to 19.4 ± 3.5% and 16.1 ± 2.9% (Fig. 2E). When N2A cells were cotransfected with mob2-shRNAs and mutant version of mob2 gene, the percentage of cells bearing neurites increased to 67.9 ± 0.4% and 68.2 ± 2.5%, respectively (Fig. 2E). Statistical analysis indicated that the number of cells bearing neurites was significantly reduced in Mob2-knockdown cells whereas

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**Fig. 3.** Mob2 promotes neuritogenesis in N2A cells. N2A cells were transiently transfected with a control pcDNA-LacZ-Myc, pcDNA-mob1A-Myc, pcDNA-mob1B-Myc, and pcDNA-mob2-Myc plasmids. After 36 h of transfection, the transfected cells were fixed, stained with anti-Myc antibody and assayed for their neurite formation. Confocal micrographs showed N2A cells expressing (A) LacZ-Myc, (B) Mob1A-Myc, and (C) Mob1B-Myc, (D) Mob2-Myc. (E) Quantitative analysis indicated that the percentage of neurite-bearing cells was 13.9 ± 0.6% in control, 15.5 ± 6.9% in Mob1A, 16.9 ± 5% in Mob1B, and 71.9 ± 1% in Mob2. Statistical analysis indicated that percentage of neurite formation was significantly higher in Mob2 expression cells. (Student’s t-test, ** indicates P < 0.001, NS indicates no significant difference). Scale bars: 50 μm.
expression of mutant version of mob2 gene in mob2-shRNA background increased the percentage of cells bearing neurites (three experiments, n > 300 neurons per experiment, Student’s t-test, \( P < 0.001 \)) (Fig. 2E). Together, these results demonstrate that the Mob2 protein plays a significant role in neurite formation in N2A cells.

### 3.3. Mob2 promotes neurite formation

We have shown that downregulation of Mob2 expression impaired neurite formation in N2A cells. We then tested whether overexpression of Mob2 promoted neurite formation in N2A cells.

In the presence of 10% serum, most N2A cells were round and remained undifferentiated. When N2A cells were transfected with the control LacZ vector, pcDNA-mob1A, and pcDNA-mob1B most cells were rounded and only a few cells bore neurites after 36 h of transfection (Fig. 3A–C). When N2A cells were transfected with pcDNA-mob2, the percentage of cells bearing neurites significantly increased after 36 h of transfection (Fig. 3D, arrows). Quantitative analysis showed that the percentage of cells bearing neurites was 13.9 ± 0.6% under the influence of the LacZ control vector, 15.5 ± 6.9% under pcDNA-mob1A, 16.9 ± 5% under pcDNA-mob1B, and 71.9 ± 1% under pcDNA-mob2. These difference were significantly in statistical analysis (three experiments, n > 300 neurons per experiment, Student’s t-test, \( P < 0.001 \)) (Fig. 3E).

Previous study has shown that overexpression of Ndr2 kinase induces neurite formation in PC12 cells [14]. To examine whether Ndr2 plus Mob2 have a synergetic effect in promoting neurite formation, N2A cells were transfected with pcDNA-lacZ, pAcGFP-ndr2, pcDNA-mob2, and pAcGFP-ndr2 plus pcDNA-mob2 respectively. In pcDNA-lacZ transfected cells, the percentage of cells bearing neurites was 8.1 ± 0.6% (Fig. 4A). In pcDNA-mob2 or pAcGFP-ndr2 transfected cells, the percentage of cells bearing neurites was 20.2 ± 0.3% or 22.4 ± 1.1%, respectively after 24 h of transfection (Fig. 4B and C). When pAcGFP-ndr2 was cotransfected with pcDNA-mob2 in N2A cells, the percentage of cells bearing neurites increased to 38.4 ± 1.5% at 24 h after transfection (Fig. 4D, arrows). To further study that expression of Ndr2 and Mob2 in promoting neuritogenesis is synergistic but not additive effect, we expressed Ndr2 in Mob2 knock-down cells and observed how it affect neuritogenesis. When pAcGFP-ndr2 was cotransfected with mob2-shRNA in N2A cells, the percentage of cells bearing neurites became 11.2 ± 2.7% at 24 h after transfection (Fig. 4E). The result is similar to LacZ control and Mob2 knock-down cells (compared to Fig. 4A and Fig. 2B). Statistics analysis indicated that the coexpression of Ndr2 and Mob2 had better effect than expression of Ndr2, Mob2, or Ndr2 in Mob2 knockdown background in neuritogenesis (three experiments, n > 300 neurons per experiment, Student’s t-test, \( P < 0.01 \), \( P < 0.001 \)) (Fig. 4F). Together, these results clearly show that Ndr2 and Mob2 have synergistic effects in promoting neurite formation in N2A cells.

### 3.4. Mob2 regulates the Moesin phosphorylation and actin rearrangement in neurite formation

Studies have shown that neurite formation is tightly modulated by Moesin phosphorylation and actin dynamics. To investigate the role of Mob2 in neuritogenesis, we decided to examine whether Mob2 regulates the expression of phosphorylated Moesin (p-Moe) and rearrangement of actin cytoskeleton. Western blot demonstrated that expression of p-Moe decreased about 50% in Mob2 knockdown cells whereas the expression of p-Moe increased 1.45 fold in Mob2 overexpression cells (Fig. 5). To further investigate whether Mob2 regulates actin rearrangement in

![Fig. 4.](image-url)
neuritogenesis, we decreased the Mob2 expression and observed the rearrangement of the actin cytoskeleton in N2A cells. In control cells, the actin filaments displayed a homogenous distribution in the cytoplasm and extended neurites (Fig. 6A). In Mob2 depleted cells, the actin filament appeared as fragments in the cytoplasm (Fig. 6B and C). When N2A cells were cotransfected with mob2-shRNAs and mutant version of mob2 gene, the fragmented actin filaments were rescued (Fig. 6D, arrowhead). Immunocytochemical results also demonstrated that expression of mob2-shRNA downregulated the expression of p-Moe (Fig. 6B and C) and the expression of p-Moe can be restored when mutant version of mob2 gene was added to the mob2-shRNA transfected cells (Fig. 6D, arrowhead). Together, these results suggest that the function of Mob2 in neuritogenesis may through regulate the expression of p-Moe and actin dynamics.

4. Discussion

How differentiated neurons extend neurites to form functional neural circuits is one of the important questions in developmental neurobiology. Studies have uncovered numerous genes that participate in neurite formation, but the molecular mechanism of this developmental process remains unclear. The function of Ndr kinases in promoting neurite formation has been found in mice [14], Drosophila [11,15], and C. elegans [16]. Biochemical studies show that Ndr kinases bind to Mob proteins, which serve as Ndr coactivators to regulate Ndr kinase activity [4,6,17,18]. In this study, we described the in vitro functions of Mob2 in differentiating neurons. We found that neurite formation was reduced significantly by using shRNAs to knockdown Mob2 expression (Fig. 2). Overexpression of Mob2 in N2A cells promoted neurite formation, but this phenomenon was not found in Mob1A and Mob1B expressing cells (Fig. 3). Based on these results, we suggest that the Mob2 protein plays an important role in neurite formation and in differentiating neurons.

The function of Mob2 in neuritogenesis is currently unclear. Mob2 belongs to the Mob superfamily protein. Studies have shown that members of this family perform different aspects of cellular functions. For example, Mob1 proteins participate in the cell cycle regulation in yeast and Drosophila [1,5] whereas the Mob2 protein induces asymmetric cell fate and promotes polarized cell growth during mitosis in Saccharomyces cerevisiae [3,9]. Furthermore, deletion of the Mob2 protein in Saccharomyces pombe results in disorganization of the actin and microtubule cytoskeleton preventing cells from growing in a polarized manner [10]. During neuritogenesis, actin filaments have been implicated in the initial sprouting of neurites whereas microtubules strengthen and support these new extensions. In our study, we showed that Mob2 proteins were localized at the initial sprouting points and the tips of neurites in differentiating neurons (Fig. 1). Downregulation of Mob2 in neuronal cells suppressed neurite sprouting in N2A cells (Fig. 2). In addition, we also observed that the rearrangement of the actin filaments was dependent on Mob2 expression (Fig. 6).

Fig. 5. Mob2 regulates the expression of phosphorylated Moein. N2A cells were transfected with mob2-shRNAs or pcDNA-mob2-Myc. After transfection, proteins were harvested, processed for SDS–PAGE, and probed with anti-p-Moe antibody. Western blot indicated the expression of p-Moe was decreased about 50% in Mob2 knockdown cells (A) but was increased to 1.45 fold in Mob2 overexpression cells (B). Statistical analysis further confirmed that p-Moe expression is decreased in Mob2 knockdown cells but is increased in Mob2 overexpression cells (three experiments, Student’s t-test, * indicates P < 0.05, ** indicates P < 0.001).
cytoskeleton was disrupted when Mob2 was downregulated in N2A cells (Fig. 6). These results suggest that Mob2 may trigger the rearrangement of the actin cytoskeleton and initiate neurite formation.

The ezrin, radixin, and moesin (ERM) proteins are family of actin-binding proteins which act as linkers between the actin cytoskeleton and plasma membrane proteins. It also serves as signal transducers in responses to cytoskeletal remodelling. Study has shown that Radixin and Moesin play important role in generating and maintaining the normal organization of growth cones [21]. Furthermore, phosphorylated ERM proteins are restricted to the filopodia of growing neurite and tether with actin filaments to regulate the dynamics of neurite protusion [22]. Our study demonstrated that expression of p-Moe was decreased in Mob2 knock-down cells and was increased in Mob2 overexpression cells (Fig. 5). Previous studies show that Mob2 interacts with Ndr2 at the N-terminal SMA (S100/MOB association) domain and then triggers the activation of Ndr2 kinase activity [17,18]. We further demonstrated that Mob2 and Ndr2 act synergistically in promoting neurite formation. Based on our observation, the possible function of Mob2 in neuritogenesis may through bind to Ndr2 and regulates the Moesin phosphorylation. Thus, it will be worth to further investigate whether Ndr2 directly phosphorylates Moesin or through other unknown proteins. In conclusion, our study provides evidence to show the importance of Mob2 in neuritogenesis. This study elucidates a novel function of Mob2 in neurite formation and provides further insights into the molecular mechanisms of neuritogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.01.003.

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