Cyr61, a Matricellular Protein, Is Needed for Dendritic Arborization of Hippocampal Neurons*

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Background: Cyr61 is an angiogenic protein with unknown neuronal function expressed in the developing nervous system.

Results: Knockdown and overexpression of Cyr61 affect dendritic arbor morphology.

Conclusion: Cyr61 is necessary to control dendritic morphology.

Significance: The function of Cyr61 in neurons has been identified for the first time.

The shape of the dendritic arbor is one of the criteria of neuron classification and reflects functional specialization of particular classes of neurons. The development of a proper dendritic branching pattern strongly relies on interactions between the extracellular environment and intracellular processes responsible for dendrite growth and stability. We previously showed that mammalian target of rapamycin (mTOR) kinase is crucial for this process. In this work, we performed a screen for modifiers of dendritic growth in hippocampal neurons, the expression of which is potentially regulated by mTOR. As a result, we identified Cyr61, an angiogenic factor with unknown neuronal function, as a novel regulator of dendritic growth, which controls dendritic growth in a β1-integrin-dependent manner.

The electrical properties of neurons are regulated by the composition and density of synapses and pattern of dendritic branching (1, 2). In mammals, the development of a proper dendritic branching pattern strongly relies on extracellular cues, such as neurotransmitters, trophic factors, the extracellular matrix, and cell adhesion molecules and the subsequent adjustment of the dynamics of intracellular processes that underlie dendrite growth and stability (3, 4). The Ras- phosphoinositide 3-kinase (PI3K) and Ras-extracellular signal-regulated kinase (ERK) signaling pathways were previously shown to contribute to brain-derived neurotrophic factor (BDNF)2-driven dendritic arborization, most likely via activation of mTOR and its downstream effector p70S6K1 (S6K1) (5). The S6K1-dependent translation of cytoskeleton-regulating proteins was shown to be sufficient to drive axonal-regulating proteins (6). It remains unknown, however, whether a similar situation occurs during dendrite growth or whether other mTOR-dependent cellular processes are also required.

For many years, mTOR and its homologs (TORs) were considered solely regulators of protein synthesis. Several reports, however, indicated a major role of TOR in the regulation of transcription. Yeast TOR1 is known to control transcription driven by all three RNA polymerases (7). In Drosophila melanogaster, large scale profiling provided evidence that dTOR controls the expression of dozens of genes (8). Research in mammals identified transcription factors, the activity or expression of which are regulated by mTOR (e.g. YY-1 (yin-yang 1), sterol regulatory element-binding protein, Maf1, and HIF1α (hypoxia-inducible factor-1α)) (9–12). YY-1, sterol regulatory element-binding protein, and HIF1α were also shown to play important roles in physiological or pathological neuronal transmission (13–15). Moreover, the transcriptional profiling of cells with increased mTOR signaling, like neuroepithelial cells that lack TSC2 or subependymal giant-cell astrocytomas derived from tuberous sclerosis patients revealed differences in the expression of several genes that are important for neuronal development (16, 17).

Matricellular proteins are secreted proteins that are closely associated with the extracellular matrix. Rather than being structural components of the extracellular matrix, however, such proteins contribute to cell signaling by acting as modulators of cell surface receptors. For example, Cyr61 (cysteine-rich 61; CCN1), a member of the CCN (Cyr61/CTGF/NOV) family of matricellular proteins, is a ligand of different integrins (18–20). Through these interactions it regulates cell adhesion, migration, proliferation, survival, apoptosis, differentiation, gene expression, and senescence, depending on the cell type and particular integrin involved (21, 22). Although Cyr61 is mostly known for its roles in various non-neuronal cells (21), it is also expressed in the developing nervous system and neuron-like cells (23, 24). Nonetheless, knowledge about the function of this protein in neurons is very limited. Thus far, the only functional data have come from the work of Kim et al. (24), who showed that knockdown of Cyr61 decreased the etoposide-in-
duced death of immortalized hippocampal progenitor H19-7 cells, suggesting a role of Cyr61 in neuronal cell death. In addition to this observation, the function of Cyr61 in neurons, especially during their development, is unknown, but several factors that induce cyr61 expression and transcription factors that regulate cyr61 transcription are known for their contribution to neuronal development and plasticity (see below).

cyr61 is an immediate early gene, the transcription of which is induced by numerous stimuli, including trophic factors (e.g., epidermal growth factor (EGF) and fibroblast growth factor (FGF)) (24, 25), Wnt (26), hypoxia (27), and mechanical stretch (28). The neuronal expression of cyr61 has not been studied thoroughly, but the activity of the cyr61 promoter was detected in the developing central nervous system of reporter mouse embryos that carried the β-galactosidase (β-gal) coding sequence under the cyr61 promoter (23). It has also been shown that in H19-7 cells, cyr61 expression can be induced by neurotransmitter glutamate, N-methyl-D-aspartate (NMDA), an agonist of NMDA-type glutamate receptors and proapoptotic drug etoposide (24). Etoposide induced cyr61 transcription, activating c-Jun N-terminal kinase (JNK) and serum response factor (SRF). Besides these observations, the regulation of cyr61 transcription in neurons under more physiological conditions remains rather unexplored.

In this work, we first showed that S6K1, the best known mTOR effector, is not sufficient to induce dendritic arbor development. Consequently, we performed short hairpin RNA (shRNA) screening to identify genes that are involved in the regulation of dendritic arbor shape, the expression of which is potentially regulated by mTOR. In this screen, we identified cyr61, which is expressed in developing neurons in a trophic factor-, Ras-, ERK-, and PI3K-dependent manner. Knockdown of Cyr61 prevented dendritic growth under basal conditions and dendritic growth induced by insulin, Ras, and PI3K. We also showed that overexpressed Cyr61 induces dendritic growth in a β1-integrin-dependent manner.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Drugs**—The mouse anti-Tau (PHF-1) antibody was described previously and kindly provided by Dr. Davies (29). The following antibodies were obtained from commercial sources: rat anti-HA (Roche Applied Science), mouse anti-α-tubulin, mouse anti-MAP2 (Sigma), rabbit anti-GFP (Medical and Biological Laboratories, Woburn, MA), rabbit anti-phospho-S6 (Ser-235/236), rabbit anti-MAP2 (Cell Signaling Technology, Danvers, MA), mouse anti-β-galactosidase (Promega, Madison, WI), mouse anti-GAD67 (Millipore, Billerica, MA), mouse anti-Tau (Tau-1) (Cedarlane, Burlington, Canada), hamster anti-CD29 (β1-integrin chain), and isotype IgM (BD Biosciences). Anti-mouse or anti-rabbit secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 568, and horseradish peroxidase (HRP) were obtained from Invitrogen and Jackson ImmunoResearch (West Grove, PA), respectively. Wortmannin and rapamycin were obtained from Calbiochem. Insulin, BDNF, bicuculline, and actinomycin D were purchased from Sigma. U0126 was purchased from Promega.

**DNA Constructs**—The following mammalian expression plasmids have been described previously: pSuper vector (30), β-actin-GFP (encoding EGFP under control of the β-actin promoter), p110CAAX, GW1-HA-RasV12, GW1-HA-RasV12S35, GW1-HA-RasV12S40, pSuper-mTOR7513 (5), β-actin-mRFP (encoding mRFP under the control of the β-actin promoter) (31), EFα-β-gal (32), and pRK5-Myc-S6K1T389E (33). The β-actin-TdTomato, encoding TdTomato under control of the β-actin promoter, was obtained by subcloning TdTomato coding sequence (34) to modified β-actin-16pl (35). The sequences that encode the shRNAs used in the screen were cloned into pSuper and are listed in **supplemental Table 1**. Scrambled shRNAs were designed based on the original Cyr61 siRNA sequences using the online siRNA Wizard version 3.1 tool and cloned into pSuper vector. The following sequences were used: 5'-GATGTAAGTCATCCTACTCTC-3' (Cyr61scr#1); 5'-GGGTGAGATCATGAGAGAA-3' (Cyr61scr#2); 5'-AGCTCTTAGGTTGTATA-3' (Cyr61scr#3). The coding sequence of cyr61 was obtained by PCR using RAT2 cell cDNA, and the product was cloned into pET12 (Fermens, Burlington, Canada) according to the manufacturer’s instructions. The pJET-Cyr61 plasmid was next used for the preparation of a template for the cRNA probe with the use of the following primers that contained promoter sequences for T3 or T7 RNA polymerases on their ends: cyr61T7 (5’-ATAAGCGTACCTATATAAGGATGAGCTCCACCACTCAAGAC- GCTCCTGTCGC-3’ and cyr61T3 (5’-AATTAACCCCTA- CTAAAGGTTGCTTGCATCTGTTGACAGACAC- AGACTG-3’). plet-Cyr61 was also used as a PCR template to obtain Cyr61 coding sequence for preparation of β-actin-Cyr61-GFP. The obtained PCR product was cloned to EcoRI and Sall sites of β-actin-GFP with modified multicloning site.

**Cell Cultures, Transfection, and Drug and Antibody Treatments**—The animals used to obtain neurons for tissue cultures and RNA from brain extracts were sacrificed according to the protocols approved by the First Ethical Committee (Warsaw, Poland). Primary hippocampal and cortical cultures were prepared from embryonic day 19 (E19) rat brains and transfected using the Lipofectamine2000 (Invitrogen) or Amaxa nucleofection procedure as described recently (36, 37). For the shRNA library screening, the neurons were transfected with pools of plasmids that encode shRNAs that target a given gene and GFP-encoding vector in the following proportions: 2:2:3 (shRNA#1/#2/#3/GFP) or 3:3:3 (shRNA#1/#2/GFP, in the case that only two shRNAs were used to silence a given mRNA). For insulin-induced dendritic growth, immediately after transfection, the neurons were transferred to a regular culture medium that contained a reduced concentration of B27 (0.2% instead of 2%; Invitrogen). Insulin (400 nm) was added for the first 4 h after transfection and then every 24 h until cell fixation. To inhibit the β1-integrin receptor, the neurons were treated with anti-CD29 antibody or isotype control twice (2 and 4 days after transfection) at a concentration of 20 μg/ml. To induce cyr61 expression, hippocampal neurons were treated with BDNF (100 ng/ml), insulin (400 nm), and bicuculline (50 μM) for 1 h. Neurons on day in vitro 8 (DIV8) prior to stimulation were kept overnight in a culture medium with 0.2% B27. DIV21 neurons were treated with these drugs after a 4-h incubation in a culture medium without B27. Actinomycin D (1 μg/ml) U0126 (20 μM) was used 48 h prior to stimulation.
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µM), wortmannin (1 µM), or DMSO (control) was added to the medium 45 min prior to insulin or BDNF.

RAT2 cells were cultured as described previously (36). To obtain high transfection efficiency, 2.2 × 10⁶ RAT2 cells were suspended in 100 µl of phosphate-buffered saline (PBS) and nucleofected with 6 µg of DNA using an Amza Nucleofector II Device (Lonza, Koln, Germany; X-005 program).

shRNA Library Screen—Hippocampal neurons were transfected on DIV8 with pools of shRNAs that targeted the chosen mRNA as described above. Four days after transfection, the neurons were fixed with 4% paraformaldehyde and 4% sucrose in PBS and immunostained for GFP. Each culture plate contained control variants transfected with pSuper or plasmid that encoded mTors shRNA. To avoid variability caused by differences between cultures, the total number of dendritic tips (TNDT) was quantified as a percentage of the mean value obtained for neurons transfected with empty pSuper from the same experimental plate. Two independent screening experiments were performed, and the mean value was calculated from both of them for each shRNA pool.

Immunocytochemistry of Hippocampal Neurons—For the immunofluorescent staining of P-S6, the neurons were fixed with 4% paraformaldehyde that contained 4% sucrose in PBS for 10 min at room temperature. Afterward, staining was performed according to the manufacturer’s protocol (Cell Signaling Technology). The immunostaining of MAP2, Tau, GAD67 and transfected proteins was performed as described previously (36).

RNA Isolation and Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)—RNA from cultured neurons and rat hippocampi was isolated with the RNeasy Protect minikit (Qiagen). cDNA was prepared with High Capacity RNA-to-cDNA Master Mix (Applied Biosystems) according to the manufacturer’s protocol. qPCR was performed using a 7900HT real-time PCR system and TaqMan gene expression assays (Applied Biosystems) with the following TaqMan rat mRNA as described above. Four days after transfection, the neurons were fixed with 4% paraformaldehyde and 4% sucrose in PBS and immunostained for GFP. Each culture plate contained control variants transfected with pSuper or plasmid that encoded mTors shRNA. To avoid variability caused by differences between cultures, the total number of dendritic tips (TNDT) was quantified as a percentage of the mean value obtained for neurons transfected with empty pSuper from the same experimental plate. Two independent screening experiments were performed, and the mean value was calculated from both of them for each shRNA pool.

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For in situ fluorescent hybridization, the cells were fixed in 4% paraformaldehyde for 10 min at room temperature and incubated with 1% H₂O₂ to minimize background. After washing with PBS that contained diethylpyrocarbonate, the cells were first incubated for 1 h at room temperature with prehybridization buffer (50% (v/v) formamide, 4 µg/ml Escherichia coli tRNA, 2% (v/v) Denhardt’s solution (Sigma-Aldrich), 1% (v/v) salmon sperm DNA (Sigma-Aldrich), and 2 mM ribonucleoside vanadyl complexes (Sigma-Aldrich)) and then with hybridization buffer (2X SSC, 50% (v/v) formamide, 10% dextran sulfate, 4 µg/ml E. coli tRNA, 2% (v/v) Denhardt’s solution, 1% (v/v) salmon sperm DNA, and 2% (v/v) ribonucleoside vanadyl complexes) that contained 1.5 ng/µl of heat-denatured digoxigenin-labeled single-stranded sense or antisense RNA probes for 10 h at 56 °C. After the hybridization step, the cells were rinsed in 2X SSC, washed once in 2X SSC for 1 h at 56 °C, washed twice in 0.2X SSC for 30 min at 56 °C, and washed four times in 0.2X SSC for 30 min at room temperature. The immunofluorescent detection of digoxigenin-labeled probes was then performed using HRP-conjugated anti-digoxigenin antibody (Roche Applied Science) and the Tyramide Signal Amplification (TSA) plus Cyanine 5 System (PerkinElmer Life Sciences). For fluorescent in situ hybridization (FISH), we used cRNA probes synthesized and labeled using the digoxigenin RNA labeling Kit (SP6/T7) (Roche Applied Science) and T3 polymerase (Roche Applied Science). The full coding sequence of cyr61 was obtained by PCR using pJet-Cyr61 and used as a template. The primers used for the PCR contained promoter sequences for T3 or T7 RNA polymerases on their ends.

Image Analysis and Quantification—Confocal neuron images were obtained with a Zeiss LSM5 Pascal or LSM710NLO microscope at 1024 × 1024-pixel resolution for morphological analysis. The ×10, ×20, and ×40 objectives were used. Each image consisted of a series of z-stack images. The resultant stack was flattened into a single image using maximum projection. The confocal settings were constant for all of the scans when fluorescence intensity was compared. Morphometric analysis and quantification were performed with MetaMorph image analysis software (Universal Imaging Corp., Downingtown, PA) for the manual counting of total number of dendritic tips and ImageJ software with the NeuronJ plug-in (38) and Sholl plug-in (available on the University of California, San Diego, The Ghosh Lab Web site) for measurements of total dendrite length and Sholl analysis, respectively. Briefly, we used the tracking function in NeuronJ to manually draw a mask of all dendrites of the transfected neuron on a confocal image and measured their length. If more than one transfected cell was present at the particular image, the mask was created only for a single cell. The mask was saved as a separate image and used in the automated Sholl analysis to avoid background of axons and other cells whose fragments could be present in the original pictures. The original script of the Sholl plug-in was modified for more accurate performance with masks instead of raw images. Images of axonal arbors were obtained with a Nikon Eclipse 80i microscope equipped with a ×10 objective, monochromatic Evolution VF charge-coupled device camera (Media Cybernetics, Silver Spring, MD), and ImageProPlus software (Media Cybernetics). For the analysis of the total length of the axons, ImageJ software with the NeuronJ plug-in was used. Dendrites and axons were primarily distinguished by morphological criteria (i.e., “short” neurites with decreasing diameter, splitting with narrow splitting angles (usually smaller than 90°) were treated as dendrites, whereas “long” neurites with relatively constant diameter splitting with variable splitting angles (often equal to or larger than 90°) were scored as axons). The additional criterion helpful for discrimination between dendrites and axons was the presence of dendritic filopodia and dendritic spines. The accuracy of morphology-based classification was additionally confirmed in selected cases (Cyr61 knockdown, Cyr61 overexpression, S6K1T389E overexpression) with immunofluorescent staining for MAP2 (marker of dendrites), Tau-1 (marker of axons of DIV0–3 neurons), and PHF-1 (marker of axons of >DIV5 neurons), as suggested previously (39). For the measurements of mean fluoresc-
cence intensity, MetaMorph image analysis software was used. Immunofluorescence intensities were measured as described previously (33). In the case of FISH analysis, the mean fluorescence signal from transfected cells was standardized to the signal from non-transfected cells from the same field of view.

**Statistical Analysis**—The data were obtained from at least three batches of cells, with the exception of the screening experiment that was performed on two batches of neurons. The exact numbers of neurons and examined for the morphology analyses are provided in the respective figure legends and in supplemental Table 2. The statistical analyses were performed using Prism (GraphPad, San Diego, CA). The Kruskal-Wallis test followed by Dunn’s post hoc test, a two-way ANOVA test followed by Bonferroni’s post hoc test, a one-way ANOVA test followed by Tukey’s multiple comparison test, the Mann-Whitney test, a paired t test, or a one-sample t test was used, depending on the type of data analyzed.

**RESULTS**

**Overexpression of S6K1 Is Not Sufficient to Induce Dendritic Growth**—We previously showed that S6K1 is needed for the development of the dendritic arbor of hippocampal neurons in dissociated culture and hippocampal slices (5). To investigate whether S6K1 activation is sufficient to induce dendritic growth, DIV8 hippocampal neurons were transfected with a plasmid that encoded a hyperactive mutant of S6K1 (S6K1T389E). Transfection with plasmids that encoded β-gal and constitutively active PI3K (p110CAAX) served as negative and positive controls, respectively. In all of the variants, the plasmid that encoded GFP was cotransfected to visualize the morphology of transfected cells (Fig. 1A). The cells were fixed on DIV14. The analysis of the TNDT revealed no differences between neurons that overexpressed β-gal and S6K1T389E (Fig. 1B), p110-CAAX, in contrast, caused a 60% increase in dendritic tips (Fig. 1, A and B) as described previously (5, 40), demonstrating the capability of the tested neurons to express dendritic arbor expansion. A TNDT is a very simplified measure that potentially could miss some subtle changes in dendritic arbor morphology. Therefore, we performed analysis of additional parameters that describe dendritic arbor morphology (i.e., measurement of total dendritic length (TDL) and Sholl analysis). Sholl analysis (41), which measures the number of dendrites that cross circles at various radial distances from the cell soma, describes changes in dendritic arbor complexity and the area of a dendritic field. Downward and leftward shifts of the Sholl plot correspond to the decreased complexity and shrinkage of dendritic arbors. Upward and rightward shifts, in contrast, reflect the increased complexity and expansion of the dendritic field. As shown in Fig. 1, C and D, also such additional analyses did not reveal an effect of S6K1T389E overexpression on proper dendritic arbor morphology. On the other hand, S6K1T389E was able to induce the phosphorylation of its downstream effector, ribosomal protein S6, on serines 235 and 236 (Fig. 1, E and F). S6K1T389E was also sufficient to induce axonal growth (Fig. 1, G and H) when transfected in DIV0 neurons for 3 days, as described previously for other hyperactive S6K1 mutants (6). These experiments demonstrated that S6K1T389E was biologically active in our preparations. Thus,
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we conclude that S6K1 activity is not sufficient to drive dendritic growth, unlike in the case of axonal development.

Several Genes, the Expression of Which Potentially Depends on mTOR, Are Needed for Proper Dendritic Arbor Morphology—Because S6K1 was not sufficient to drive dendritic growth, we searched for new potential mTOR effectors needed for this process. Recent phosphoproteomic studies identified several regulators of transcription and mRNA processing among mTOR substrates (43, 44). Therefore, we decided to test whether the genes that encode mRNAs whose levels were previously shown to depend on mTOR activity (8, 16, 17) are involved in dendritic arbor development. To achieve this, we designed a minilibray of shRNAs that target 46 such mRNAs (supplemental Table 1) using algorithms that proved to be accurate in over 66% of the cases in our previous studies (i.e. two of three designed shRNAs were effective) (5, 36, 37). These shRNAs were cloned into pSuper vector. Hippocampal neuronal cells were then transfected on DIV8 with pools of shRNAs that targeted each selected mRNA for 4 days. The plasmid that encoded GFP was cotransfected to help identify transfected cells and visualize their morphology. Two control variants were included on each experimental plate. As a negative control, we used cells transfected with empty pSuper. As a positive control, we transfected cells with pSuper-mTOR7513 (5). To assess changes in dendritic arbors, we quantified TNDT. As expected from our previous studies (5), transfection with pSuper-mTOR7513 caused a drop in TNDT compared with pSuper-transfected neurons (Fig. 2; also see supplemental Table 2). Several tested pools of shRNAs (16 of 46) caused a significant decrease in TNDT (p < 0.01) in each of the two experiments (for details, see supplemental Table 2). For these pools, the effects ranged from a 23% to a 72% decrease in TNDT compared with the negative control (supplemental Table 2). Among the positive shRNA pools, we decided to focus on one that targeted mRNA that encoded matricellular protein Cyr61 for further functional evaluation.

Cyr61 Expression in Neurons—The expression and function of Cyr61 have not been previously studied in developing neurons. Therefore, before going into more detail of the involvement of Cyr61 in dendritic arborization, we decided to better characterize the cyr61 expression profile in neurons. First, we determined the levels of cyr61 mRNA at different developmental stages using RT-qPCR, both in our in vitro preparations and in vivo. As shown in Fig. 3A, the expression of cyr61 mRNA was higher in still-developing hippocampal neurons (DIV7 and DIV10) than in mature hippocampal neurons (DIV14 and DIV17). In vivo, with the use of RNA isolated from the hippocampi of E18, P5, P10, P15, and P30 rats, we observed a significant decrease of cyr61 expression between E18 and the postnatal period (Fig. 3B). However, differences observed during the postnatal period, although following a trend similar to cells maturing in vitro, did not reach statistical significance (Fig. 3B).

cyr61 is an immediate early gene, the expression of which is potentiated by several stimuli (see above). The results of our shRNA library screen prompted us to investigate whether BDNF and insulin, two factors important for dendritic arbor development (5, 33), induce higher cyr61 mRNA levels in developing neurons. To achieve this, DIV8 cells were first B27-starved overnight to equalize cellular responses and then treated with insulin (400 nM) or BDNF (100 ng/ml) for 1 h. As shown in Fig. 3, C and D, both insulin and BDNF increased cyr61 mRNA levels. Pretreatment with actinomycin D (ActD), a transcription inhibitor, prevented these increases (Fig. 3, C and D), confirming that cyr61 expression in our model undergoes transcriptional regulation. Interestingly, cyr61 expression could still be induced, despite very low basal levels, in mature DIV21 neurons by insulin, BDNF, and 50 μM bicuculline, an antagonist of γ-aminobutyric acid A receptors that are responsible for inhibitory transmission (Fig. 3E).

Receptors for insulin and BDNF act via two major signaling pathways, PI3K and ERK. Consequently, we tested whether both pathways are needed for the insulin- and BDNF-induced transcription of cyr61. As shown in Fig. 3, F and G, pretreatment of neurons with wortmannin, an inhibitor of PI3K, prevented the increase in cyr61 mRNA levels in both cases. Pretreatment of neurons with UO126, an inhibitor of MEK1/2, prevented the increase in cyr61 mRNA levels in response to BDNF (Fig. 3G). Also, in the case of insulin stimulation, we observed a moderate inhibitory effect of UO126, which, however, did not reach statistical significance (Fig. 3F). There were no statistically significant differences between the following variants: ActD and ActD/insulin, ActD and ActD/BDNF,

FIGURE 2: shRNA screening results indicate that Cyr61 is a possible mTOR effector involved in dendritogenesis. Shown are the results of screening for possible mTOR effectors involved in dendritogenesis. Hippocampal neurons cultured in vitro were transfected on DIV8 for 4 days with pSuper vectors that encode pools of two or three shRNAs that target mRNAs possibly regulated by mTOR at the level of transcription. A GFP-encoding plasmid was cotransfected for the visualization of transfected neurons. As controls, empty pSuper or pSuper-mTOR7513 (encoding mTORsh) was transfected to neurons on each culture plate. The plot represents the mean TNDT ± S.E. (error bars) obtained in two independent experiments, expressed as a percentage of a corresponding pSuper-transfected negative control from the same culture plate.
UO126 and UO126/insulin, wortmannin and wortmannin/insulin, UO126 and UO126/BDNF, and wortmannin and wortmannin/BDNF. Both PI3K and MEK1/2 can stimulate mTOR activity. To provide evidence of the dependence of cyr61 transcription on mTOR, we treated DIV8 neurons with 20 nM rapamycin for either 1 or 3 h. Because of the typical response of immediate early genes to protein synthesis inhibitors, cyr61 mRNA levels dramatically increased (data not shown), precluding further pharmacological analysis of the direct effects of mTOR on cyr61 transcription. Thus, we concluded that the expression of cyr61 in response to BDNF stimulation depends on PI3K and ERK signaling, whereas insulin-dependent cyr61 expression relies more strongly on PI3K in neurons.

Cyr61 Is Indispensable for Proper Dendritic Arbor Morphology of Developing but Not Mature Neurons under Basal Culture Conditions—Once we confirmed that cyr61 is expressed in hippocampal neurons cultured in vitro at the time of intensive dendritogenesis and that it is regulated by factors that induce this process, we then returned to the analysis of the effects of Cyr61 on the dendritic arbors of hippocampal neurons. We first confirmed that our Cyr61 shRNAs down-regulated cyr61 mRNA. To achieve this, we first nucleofected DIV0 cortical neurons with pSuper, pSuper-Cyr61sh#1, -sh#2, or -sh#3 or a pool of all three plasmids. However, the transfection of such young neurons with shRNAs against Cyr61 was lethal (data not shown). Therefore, we decided to use a non-neuronal rat cell line and nucleofected Cyr61 shRNAs to RAT2 cells. As shown in Fig. 4A, the pool of Cyr61 shRNAs as well as Cyr61sh#2 and -sh#3 effectively decreased the levels of endogenous cyr61 mRNA measured with the use of RT-qPCR 48 h after nucleofection. Cyr61sh#1 also decreased Cyr61 mRNA, but this decrease did not reach statistical significance (but see Fig. 5).

For further verification of the specificity of a phenotypic effect caused by our Cyr61 shRNAs, we used a battery of standard approaches: (i) shRNA pool separation, (ii) scrambled shRNA overexpression, and (iii) phenotype “rescue” (45). Regarding the first approach, we transacted DIV8 neurons for 4 days with pSuper that encoded Cyr61sh#1, -sh#2, or -sh#3. As
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FIGURE 4. Knockdown of Cyr61 in developing neurons simplifies dendritic tree morphology. A, Cyr61shRNA validation was performed after nucleofection of RT2 cells with pSuper, Cyr61sh#1, -sh#2, or -sh#3 or a pool of shRNAs. The cells were lysed 2 days after nucleofection, and Cyr61 mRNA levels were assessed by RT-qPCR, quantified relative to GAPDH, and compared with pSuper-nucleofected controls. The plot represents the mean values ± S.E. (error bars) from four independent experiments. ***, p < 0.01; *, p < 0.05, compared with control value = 1 (one-sample t test); ns, not significant. B, representative images of cultured in vitro rat hippocampal neurons transfected on DIV8 for 4 days with pSuper vector (control) or plasmids that encode individual Cyr61sh or their pool (Cyr61sh-mix). The GFP-encoding plasmid was cotransfected for the visualization of transfected cells. Scale bar, 50 µm. C, TNDT of neurons transfected as described in B. Cell images were obtained from three independent culture batches. The data are expressed as mean values ± S.E. (pSuper, n = 53; Cyr61sh-mix, n = 58; Cyr61sh#1, n = 51; Cyr61sh#2, n = 42; Cyr61sh#3, n = 47). ***, p < 0.001, compared with pSuper (Kruskal-Wallis test followed by Dunn’s post hoc test). D, TNDT of neurons transfected with either pSuper or scrambled shRNA vector and GFP-encoding plasmid as described in B. Cell images were obtained from three independent culture batches (pSuper, n = 54; Cyr61scr#1, n = 55; Cyr61scr#2, n = 45; Cyr61scr#3, n = 51). The data are expressed as mean values ± S.E. ns, not significant (compared with pSuper; Kruskal-Wallis test followed by Dunn’s post hoc test). E, TNDT of neurons transfected with either pSuper or scrambled shRNA vector and GFP-encoding plasmid as described in B. Cell images were obtained from three independent culture batches (pSuper, n = 54; Cyr61scr#1, n = 55; Cyr61scr#2, n = 45; Cyr61scr#3, n = 51). The data are expressed as mean values ± S.E. ns, not significant (compared with pSuper; Kruskal-Wallis test followed by Dunn’s post hoc test; see supplemental Table 3 for detailed statistics for F).

A negative control, we used pSuper. As a positive control, we transfected cells with pooled Cyr61 shRNAs similarly to our initial screen (Fig. 2). Again, GFP-encoding plasmid was cotransfected for the identification of transfected cells and visualization of their morphology. As shown in Fig. 4, B and C, each of the tested Cyr61sh and their pool significantly decreased TNDT compared with pSuper-transfected cells. However, we did not observe any significant changes in TNDT when we repeated the experiments using their scrambled counterparts instead of Cyr61 shRNAs (Fig. 4D), which did not silence cyr61 expression nucleofected to RT2 cells (data not shown). Because effects of Cyr61 on dendrites were not previously reported, to additionally confirm that in case of Cyr61 knockdown our morphological criteria for dendrite selection are correct, we independently repeated experiment using either pSuper or Cyr61 shRNA pool transfection and quantified the number of MAP2-positive/Tau (PHF-1)-negative neurites. With such criteria we also showed that Cyr61 knockdown results in a significant decrease in the number of dendrites (pSuper/GFP (n = 50), 22.58 ± 0.72; Cyr61sh-mix (n = 46), 16.09 ± 0.61; p < 0.001; Mann-Whitney test). Because embryonic hippocampal cultures are mixtures of a large population of excitatory and a small population of inhibitory (glutamic acid decarboxylase (GAD67)-positive) neurons, we tested if knockdown of Cyr61 affects selectively one of them. Toward this end, we analyzed dendritic arbors of GAD67-positive cells transfected with either pSuper or Cyr61sh-mix and did not see any differences (data not shown). This suggests that Cyr61 knockdown selectively affects excitatory neurons.

The analysis of the additional parameters that describe dendritic arbor morphology also revealed the requirement of Cyr61 for proper dendritic arbor morphology. The TDL was decreased by 64, 61, and 54% for Cyr61sh#1, -sh#2, and -sh#3, respectively (Fig. 4E). These effects of individual Cyr61 shRNAs on TDL were comparable with the one obtained by the pool of shRNAs (55% reduction in TDL). We also used Sholl analysis of the dendritic fields of neurons that lack Cyr61 and confirmed their shrinkage. Specifically, the Sholl plots were strongly shifted downward and slightly leftward (Fig. 4F and supplemental Table 3). The number of maximum crosses was significantly lower (8.0 ± 0.4, 7.8 ± 0.4, 10.7 ± 0.6, and 9.1 ± 0.5 for the Cyr61sh#1, -sh#2, -sh#3, and Cyr61sh pool, respectively, compared with 15.7 ± 0.5 for pSuper) and reached closer to the cell soma in Cyr61 shRNA-transfected cells (pSuper, 40 µm; Cyr61sh#1, -sh#2, -sh#3, and the Cyr61sh pool, 30 µm; Fig. 4E).

To unequivocally confirm the specificity of the observed effects of Cyr61 knockdown on dendritic arbor morphology, we “rescued” the Cyr61 knockdown phenotype overexpressing GFP-tagged Cyr61. We took advantage of the fact that Cyr61sh#3 targets the 3’-untranslated region (UTR) of endogenous rat Cyr61 mRNA, which is spared in the plasmid that encodes Cyr61-GFP (Fig. 5A). Indeed, as shown in Fig. 5B, when we transfected DIV8 neurons for 4 days with Cyr61-GFP and either pSuper or pSuper-Cyr61sh#3, we did not observe substantial differences in the levels of GFP fluorescence. However, when we used pSuper-Cyr61sh#1 or -sh#2 for cotransfection with Cyr61-GFP, GFP fluorescence was greatly diminished (Fig. 5B). In all four cases, the fluorescence of mRFP, the cDNA of which was cotransfected for the visualization of transfected cells, remained intact (Fig. 5B). We next tested whether the transfection of Cyr61-GFP reverses the deleterious effects of a Cyr61 knockdown on neuronal dendritic arbors. Indeed, over-
expression of Cyr61-GFP fully reversed the negative effects of Cyr61sh#3 on TNDT (Fig. 5, C and D). As expected, 4-day overexpression of Cyr61sh#3 without the rescue construct decreased TNDT (Fig. 5, C and D). The overexpression of Cyr61-GFP alone for the same time had no significant effect on dendritic arborization (Fig. 5, C and D). Also, using analysis of TDL and Sholl analysis, we confirmed the ability of Cyr61-GFP to rescue the effects of Cyr61 knockdown (Fig. 5, E and F, and supplemental Table 3). However, similar to our previous observations, rescue of more complex parameters of a dendritic arbor was only partial (33, 36, 37). Based on the above results, we concluded that the effects of Cyr61 knockdown on dendritic arbor morphology are specific. Because the pool of shRNAs acted similarly to separate shRNAs, we decided to use only pooled shRNAs that allow the use of lower doses of individual shRNAs, thereby reducing potential off-target effects.

The experiments described thus far suggested that the expression of cyr61 is relatively high in developing neurons and needed for proper dendritic arborization at this stage. The expression of cyr61 significantly dropped at the time of neuronal maturation and dendritic arbor stabilization. Therefore, we tested whether the morphology of the dendritic trees of mature neurons that express low levels of Cyr61 still rely on this protein. To achieve this, DIV15 neurons were transfected with the Cyr61 shRNA pool for 4 days (Fig. 6A). Neurons transfected with pSuper were used as a control. Similar to the previous experiments, GFP was cotransfected for neuronal morphology visualization. In contrast to developing neurons, Cyr61 knockdown in mature cells did not cause any differences in TNDT or TDL compared with the control (Fig. 6, A–C). The Sholl analysis did not reveal any major differences in the dendritic fields of these cells (Fig. 6D and supplemental Table 3). Thus, we concluded that Cyr61 is needed for the proper dendritic arbor morphology of developing but not mature neurons under basal culture conditions.

Cyr61 Knockdown Prevents Insulin-, Ras-ERK-, and Ras-PI3K-driven Dendritic Arborization—We identified Cyr61 in a screen of mTOR-dependent proteins required for dendritic arborization and found that cyr61 expression is controlled by insulin and the PI3K and ERK pathways. mTOR activity was previously shown to be needed for the induction of dendritic growth of developing neurons both by insulin (33) and overexpression of constitutively active Ras mutants that specifically activate ERKs (RasV12S35) and PI3K (RasV12C40) (5). Therefore, we tested whether Cyr61 is needed for dendritic arborization in three independent models of mTOR-dependent growth established previously in our laboratory, namely chronic insulin treatment under B27-reduced cultured conditions (33), overexpression of constitutively active Ras mutants (5), and overproduction of constitutively active PI3K (5, 36). We first used our pharmacological model of dendritic arbor growth (33). To determine whether Cyr61 is essential for insulin-induced growth, we compared the dendritic arbors of neurons transfected on DIV8 with pSuper or a pool of shRNAs that target Cyr61, starved of B27, and chronically treated with insulin. A GFP-encoding vector was used to visualize the morphology of transfected cells. As shown in Fig. 7, A–C, the transfection of the Cyr61 shRNA pool resulted in lower TNDT and TDL in response to insulin compared with pSuper-transfected controls. In fact, these measures were identical when compared with non-stimulated neurons transfected with the Cyr61
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FIGURE 6. Cyr61 knockdown in mature neurons does not disrupt dendritic tree morphology. A, representative images of rat hippocampal neurons cultured in vitro and transfected on DIV15 for 4 days with pSuper vector (control) or a pool of plasmids that encode Cyr61sh#1, -sh#2, and -sh#3 (Cyr61sh-mix). GFP-encoding plasmid was cotransfected for the visualization of transfected cells. Scale bar, 50 μm. Shown are TNDT (B), TDL (C), and Sholl analysis (D) of neurons transfected as in A. Cell images were obtained from three independent culture batches (pSuper, n = 36; Cyr61sh-mix, n = 32). The data are expressed as mean values ± S.E. (error bars). ns, not significant (Mann-Whitney test for B and C; two-way ANOVA test followed by Bonferroni’s post hoc test in D).

shRNA pool. Similar results were obtained with Sholl analysis (Fig. 7D and supplemental Table 3).

We then determined the importance of Cyr61 for dendritic growth in our two genetic models of induced dendritogenesis. In the first case, we transfected DIV8 neurons with the constitutively active Ras mutants RasV12, RasV12S35, and RasV12C40 for 5 days and observed a substantial increase in TNDD in all three cases compared with control cells transfected with EFα-β-gal (Fig. 8, A and B). When any of the Ras mutants were expressed together with the Cyr61sh pool, Ras-induced growth was completely blocked and not significantly different from cells transfected with β-gal and Cyr61 shRNA pool (Fig. 8, A and B). On the other hand, overexpression of Cyr61scr pool did not prevent Ras-induced dendritic arborization (Fig. 8C).

To obtain more detailed insight into changes of dendritic arbor caused by Ras overexpression combined with Cyr61 knockdown, we performed TDL and Sholl analyses. As shown in Fig. 8D, overexpression of Ras mutants potently increased TDL, and this effect was blocked by knockdown of Cyr61. As shown previously (5), overexpression of Ras mutants caused upward and rightward shifts of Sholl plot, and these effects were blocked by cotransfection of Cyr61 shRNA pool (Fig. 8E and supplemental Table 3). Altogether, our data suggest that Cyr61 is indeed needed for Ras-induced dendritic growth that relies on either ERK or PI3K. To further corroborate this conclusion, we tested whether constitutively active Ras and its more specific variants are capable of increasing cyr61 expression. To achieve this, we nucleofected freshly isolated cortical neurons (37) with a constitutively active mutant of Ras (RasV12) and its variants that selectively activate either the PI3K (RasV12C40) or ERK (RasV12S35) pathway (5). All three mutants were effectively expressed in neurons upon nucleofection and increased the amount of cyr61 mRNA compared with control neurons transfected with EFα-β-gal (Fig. 8, F and G). It should be noted, however, that the Ras mutant expression levels were not equal (Fig. 8F).

We previously showed that RasV12S35 and RasV12C40 control neuronal development in an mTOR-dependent fashion (5). Therefore, in a last series of experiments, we focused on PI3K-induced dendritic arborization, which is well documented to depend on mTOR (5, 40). DIV8 neurons were transfected with p110CAAX or EFα-β-gal (i.e. a negative control) together with pSuper or the pool of Cyr61sh for 5 days (Fig. 9A). We observed a substantial increase in TNDT and TDL of p110CAAX-transfected cells compared with control cells transfected with EFα-β-gal (Fig. 9, A and B). Also, the Sholl plot was shifted upward and rightward (Fig. 9C and supplemental Table 3). However, when p110CAAX was expressed together with the Cyr61sh pool, PI3K-induced growth was blocked (Fig. 9, A–C). On the other hand, when we used the Cyr61 scrRNA pool, the PI3K-induced growth was sustained (data not shown). Using nucleofection, we showed that RasV12C40, which should selectively induce only the PI3K pathway, increases cyr61 mRNA levels in young neurons. We could not use this approach, however, for p110CAAX because of the poor expression of p110CAAX after nucleofection with the Amaxa system (data not shown). Thus, to prove that p110CAAX overexpression in neurons can increase cyr61 mRNA levels, we transfected DIV8 hippocampal cells in culture with Lipofectamine2000 and performed FISH to detect cyr61 transcripts. As shown in Fig. 9E, our antisense Cyr61 probe gave a higher signal than the sense one. However, p110CAAX overexpression for 2 days was able to further increase cyr61 mRNA levels in transfected cells compared with EFα-β-gal-transfected control neurons or surrounding non-transfected cells (Fig. 9, F and G). Therefore, we concluded that Cyr61 is indispensable for the acquisition of proper dendritic morphology in models of mTOR-dependent dendritic growth when dendritic development is enhanced by insulin, Ras-ERK, or Ras-PI3K and that its mRNA level depends on these signaling pathways.
Cyr61 Is Sufficient to Induce Dendritic Growth of Developing and Mature Neurons—Our experiments revealed that Cyr61 is required for the proper dendritic arbor morphology of developing neurons and suggested that 4-day overexpression of Cyr61-GFP was not sufficient for the induction of dendritic growth in such neurons (Fig. 5). However, evidence was provided previously that relatively short, 3-day overproduction of constitutively active PI3K was also not sufficient to induce the expansion of dendritic arbors (5). Thus, we tested whether longer 6-day overproduction of Cyr61-GFP is able to increase dendritic arborization. First, we transfected DIV8 neurons with either β-actin-GFP (negative control) or β-actin-Cyr61-GFP (Fig. 10A). β-Actin-mRFP was added to transfection mixtures to help visualize the morphology of transfected neurons. Six-day Cyr61-GFP overexpression increased TNDT by 42% compared with GFP-overproducing cells (Fig. 10B). The TDL also increased by 26% (Fig. 10C). Finally, the Sholl plot was significantly shifted upward, starting from 20 μm until 140 μm from the cell soma (Fig. 10D and supplemental Table 3). Consequently, the number of crossings in a maximum branching point increased from 16.7 ± 0.6 to 21.6 ± 0.7 (Fig. 10D). Additionally, the maximum branching point was shifted rightward in Cyr61-GFP-overexpressing cells (50 and 70 μm for GFP and Cyr61-GFP overexpression, respectively; Fig. 10D). Similar to experiments described above for Cyr61 knockdown, we independently confirmed that Cyr61 overexpression affects dendrites, counting the number of MAP2-positive/Tau (PHF-1)-negative neurites of control and Cyr61-overexpressing neurons. Using this approach, we showed that Cyr61 overexpression results in a significant increase of dendrite number (GFP/TdTomato (n = 53), 18.98 ± 0.68; Cyr61-GFP/TdTomato (n = 40), 22.23 ± 0.63; p < 0.01; Mann-Whitney test). We also tested if Cyr61 overexpression affects GAD67-positive cells and did not see any differences when compared with control (data not shown).

Although Cyr61 appeared spare for morphology of dendritic arbors of mature neurons, we asked whether increasing Cyr61 levels in such neurons could modify already established dendritic morphology. Neurons on DIV15 were transfected similarly to DIV8 neurons and fixed 6 days later. As shown in Fig. 10, E–H, and supplemental Table 3, overproduction of Cyr61-GFP in mature cells effectively increased the number of dendritic
tips and TDL and induced the expansion of dendritic fields compared with GFP-expressing controls. Altogether, these data demonstrated that Cyr61 overexpression is capable of inducing dendritic field expansion in both developing and mature neurons.

_Cyr61 Controls Dendritic Arbor Morphology via β1-Integrin—_ How Cyr61 regulates dendritic morphology at the molecular level is currently unknown. Cyr61 is a ligand of β1-integrin (CD29) in fibroblasts (46, 47). β1-Integrin is also crucial for proper dendritic arbor morphology (48, 49). If Cyr61 acts via β1-integrin, then the effects of Cyr61-GFP overexpression should be diminished by a β1-integrin-blocking antibody. Such a strategy was efficiently used recently for studying the role of matrix metalloproteinase-9 in the dynamics of dendritic spines.
of hippocampal neurons (50). To test this hypothesis, DIV8 neurons were transfected with either a GFP- or Cyr61-GFP-encoding vector together with an mRFP-encoding vector to visualize neuronal morphology for 6 days. Two days post-transfection, the cells were treated with anti-Cy3/29 antibody (20 μg/ml). The antibody was again added 4 days after transfection. As a control, the cells were exposed to the same concentration of isotopic antibody. Six-day overexpression of Cyr61-GFP in the presence of IgM increased TNNT compared with IgM-treated, GFP-overexpressing cells (Fig. 10, I and J). However, the application of an anti-Cy3/29 antibody counterbalanced the effect of Cyr61 overexpression (Fig 10, I and J). Application of the anti-Cy3/29 antibody did not have any significant effects on control cells transfected with β-actin-GFP (Fig 10, I and J). This observation strongly implies that Cyr61 indeed regulates dendritic arborization via β1-integrin.

**DISCUSSION**

The present study showed that S6K1 is not sufficient for the control of dendritic growth as opposed to axonal development. This observation prompted us to perform shRNA screening for modifiers of the dendritic arbor, the transcription of which may depend on the mTOR pathway. As a result, the function of the matricellular protein Cyr61 in developing neurons was described for the first time. We showed that (i) cyr61 was expressed in neurons during the time of intensive developmental dendritic arbor rearrangements; (ii) cyr61 expression was increased by BDNF, insulin, Ras, and PI3K, known upstream activators of mTOR; and (iii) mTOR-dependent dendritic arborization induced by insulin, Ras, and PI3K was blocked by Cyr61 knockdown. Finally, we showed that Cyr61 overexpression induces the dendritic arborization of both developing and mature neurons, and this effect depended on β1-integrin.

We consider the observation that cyr61 is expressed abundantly at the time of intensive dendritogenesis and is needed for this process to be the most important finding of the present work. We also showed that blocking β1-integrin prevented the positive effects of Cyr61 overexpression on dendritic arborization. The importance of Cyr61 for dendritic arbor development and the proposed mechanism that involves β1-integrin are further supported by previously published results that showed that (i) different integrins, including β1-integrin, serve as Cyr61 receptors (18, 20, 46) and (ii) β1-integrin is important for dendritic arborization. Scholmann et al. (48) showed that β1-integrin is needed for semaphorin 3A-induced dendritic arborization in cultures in vitro. Recently, Warren et al. (49) confirmed the importance of this protein for the postnatal dendritic arbor development of hippocampal neurons in vivo. Interestingly, other CCN family members and angiogenic factors different from Cyr61 contribute to neuronal development. For example, CCN5 induces neurite outgrowth in a neuroblastoma Neuro2a cell line in an integrin-dependent manner (51). Licht et al. (52) showed that the angiogenic factor VEGF is necessary for the dendritic arbor development of adult-born interneurons that integrate into olfactory bulb circuitry. Another angiogenic protein, angiopoietin-1, was able to induce neurite outgrowth in PC12 cells (53) and increase the dendritic length of CA1 neurons when overexpressed in vivo (54).

An unanswered question is which signaling events contribute to dendritic growth downstream of Cyr61. Cyr61 was shown to activate small GTase Rac1 in prostate cancer cells (55), which is a widely accepted activator of dendritic growth (3, 56, 57). Cyr61 can also act upstream of PI3K-mTOR and ERK signaling pathways (58, 59), raising the intriguing possibility that the initial activation of cyr61 expression downstream of these kinases is an early step in a positive feedback loop that potentiates their progrowth effects and coordinates the actions of these kinases with remodeling of the extracellular matrix.

We previously showed that the canonical mTOR downstream target S6K1, which is involved in the control of translation, is needed for dendritic growth similarly to axonal growth (5, 6). However, the present study suggests that S6K1 activity is not sufficient to induce dendritic growth, as opposed to axonal growth. This prompted us to search for novel candidates that may be important for dendritic growth. We focused on proteins, the expression of which is potentially regulated by mTOR at the level of either transcription or mRNA stability. We chose this particular group for two reasons. First, these processes are crucial for dendritic growth (3, 4). Second, several groups reported the involvement of mTOR or its homologs in transcriptional control (8, 10, 60, 61). Our results (Fig. 2) showed that indeed several tested shRNA pools caused improper dendritic arborization. Among our positive hits, we focused on Cyr61 in this study, the physiological function of which has not been previously reported in neurons. We found that cyr61 expression was increased by several upstream regulators of mTOR, such as insulin, BDNF, Ras, PI3K, and ERKs. However,
we failed to directly prove the necessity of mTOR for cyr61 transcription. This is because cyr61 mRNA levels increased upon rapamycin treatment. This observation is consistent with the behavior of other immediate early genes, the transcripts of which are stabilized under conditions of protein translation inhibition. Thus, the potential involvement of mTOR in the control of cyr61 transcription is currently only suggested by the indirect evidence discussed above. Additional support for this hypothesis comes from published studies. For example, cyr61 expression is increased in mouse neuroepithelial cells that lack TSC2 (tuberous sclerosis complex 2) and are characterized by increased mTOR activity (17). However, we cannot exclude the possibility that the increased expression of some genes in such a model is mTOR-independent because this was not rigorously tested by the authors. Another line of reasoning derives from known mTOR links to some cyr61 transcription regulators. The transcription factors involved in the control of cyr61 expression in various cellular models include TCF4 (26), SRF (62), CBP (62), CREB (63), FOXO3a (64), AP1 (27, 63), STAT3 (65), and HIF1/2 (27). The most obvious link is HIF1/2, known as an mTOR effector induced under hypoxic conditions (9). However, HIF1/2 can be activated in an oxygen-independent manner in neurons (15). We did not observe any significant effects of overexpression of either the dominant negative or constitut-
a new pathway that regulates dendritic growth in a β1-integrin-dependent manner. The activity of two other transcription factors was shown to be regulated by the mTOR pathway in neuroblastoma cells treated with ciliary neurotrophic factor (66). The activity of these transcription factors, such as SRF (69), CBP, CREB (70), and AP1 (42) are also involved in the regulation of dendritic growth of different types of neurons, similar to mTOR and Cyr61. Thus, an intriguing question is whether these transcription factors in neurons can be regulated by the PI3K and ERK pathways, independent of mTOR. Therefore, this kinase might not be necessary for the activation of Cyr61 expression by these two signaling pathways. Thus, although the role of Cyr61 in shaping dendritic arbors is unequivocal, further studies are needed to directly prove the participation of mTOR in the control of Cyr61 expression during dendritogenesis and reveal the responsible transcription factors.

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