INTRODUCTION

Profilins are major regulators of the actin cytoskeleton. Initially, they were described as proteins which directly bind to actin.\(^1,\)\(^2\) However, actin is not the only interactor—profilins harbor a number of conserved binding sites for a plethora of proteins and phospho-lipids.\(^3,\)\(^5\) Those interactions are critical for the regulation of actin dynamics in the cellular environment. This specificity is not only mediated by its binding to actin but also its interaction with phospholipids such as phosphatidylinositol (4,5)-bisphosphate (PIP\(_2\)) at the membrane and a plethora of proteins containing poly-L-proline (PLP) stretches. These interactions are fine-tuned by posttranslational modifications such as phosphorylation. Several phospho-sites have already been identified for profilin\(_1\), the ubiquitously expressed isoform. However, little is known about the phosphorylation of profilin\(_2\). Profilin\(_2\) is a neuronal isoform important for synapse function. Here, we identified several putative profilin\(_2\) phospho-sites in silico and tested recombinant phospho-mimetics with regard to their actin, PLP and PIP\(_2\) binding properties. Moreover, we assessed their impact on actin dynamics employing a pyrene-actin polymerization assay. Results indicate that distinct phospho-sites modulate specific profilin\(_2\) functions. We could identify a molecular switch site at serine residue 71 which completely abrogated actin binding—as well as other sites important for fine-tuning of different functions, for example, tyrosine 29 for PLP binding. Our findings suggest that differential profilin\(_2\) phosphorylation is a sensitive mechanism for regulating its neuronal functions. Moreover, the dysregulation of profilin\(_2\) phosphorylation may contribute to neurodegeneration.

KEYWORDS

actin binding, actin polymerization, phosphatidylinositol, poly-L-proline, protein phosphorylation
Actin dynamics are characterized by the continuous attachment of globular (G-) actin monomers at the barbed end of the filamentous (F-) actin. Together with the dissociation of G-actin at the pointed end, this process defines the length of an actin filament. In cells, most G-actin is bound to profilins which accelerate the exchange of ADP against ATP on G-actin, thus replenishing the available pool of ATP-actin. ATP-actin displays a high affinity for the barbed end of F-actin. As a consequence, a profilin-actin dimer binds to the barbed end. F-Actin, however, has a reduced affinity for profilin leading to profilin’s dissociation. Free profilin is then ready to bind another ADP-actin monomer thus completing its functional cycle.

Binding of actin to profilin competes with its interaction to phosphoinositides such as phosphatidylinositol (4,5)-bisphosphate (PIP2) at the membrane. Thus, PIP2 binding may be used as a regulatory mechanism preventing profilin from further interactions with actin. Growth factor–induced activation of phospholipase C (PLC) is able to release profilin. The liberated profilin fraction then modulates actin dynamics at the membrane. Additionally, profilin binds simultaneously to actin and proteins with poly-L-proline (PLP) stretches. This allows PLP proteins to interfere with actin dynamics in multiple ways. PLP proteins can be classified as general modulators of actin dynamics such as formins and related proteins involved in regulating motile processes of cells. The affinity of profilins for PLP is dramatically reduced when bound to PIP2. Thus, PIP2 binding excludes that profilin interacts with actin and PLP proteins simultaneously.

These interactions with binding partners are presumed to be modulated by post-translational modifications such as phosphorylation which allows a fine-tuning of profilin activity in the cellular context. The ubiquitously expressed profilin1-isoform is phosphorylated at multiple sites, thereby affecting its PLP and actin binding properties. ROCK-mediated phosphorylation at S137 reduces PLP binding capacity and to a lesser extent actin binding ability. Phosphorylation at T89 and Y128 enhances the affinity for actin. Profilin2a is the only isoform predominantly expressed in CNS tissue. Similar to profilin1, profilin2a is a binding partner of the Rho-kinase (ROCK) indicating that phosphorylation modulates its activity. To our knowledge, the consequences of profilin2a phosphorylation for actin, PLP and PIP2 binding have not been characterized so far. A better understanding of the molecular properties of profilin2a is important to complement our view of the dynamic processes in neuronal systems. This becomes particularly important for the concept of balanced neurotransmitter homeostasis since profilin2a is involved in regulating synaptic PLP proteins of the intracellular membrane trafficking pathways.

Here, we comprehensively characterized several putative profilin2a phospho-sites and dissected their impact on distinct molecular functions and properties. Accordingly, we purified recombinant phospho-mimetics which we employed in vitro to investigate their thermal stability, binding to PIP2, PLP, and actin. In particular, we assessed the effect of profilin2a phospho-mimetics on the dynamics of actin polymerization. Our results reveal that phosphorylation of profilin2a at specific sites can serve as a molecular mechanism to modulate various cytoskeleton-related functions in cells.

## 2 MATERIALS AND METHODS

### 2.1 In silico analysis and selection of phospho-sites

Putative phospho-sites of human profilin2a (NCBI reference sequence NP_444252.1) were determined using the NetPhos 3.1 Server. Seven predicted sites were chosen for further analysis. The selection of these phospho-sites was based on their position relative to interaction sites. This was determined with the molecular graphics-program CCP4mg.

Due to the very high sequence similarity between human and mouse profilin2a, we used the available X-ray structure of mouse profilin2a in complex with the proline-rich domain of VASP (RCsb PDB: 2V8C). Amino acid residues involved in actin and PIP2 binding were determined by comparison with known residues for mammalian and amoeba profilins.

### 2.2 Protein purification

Human profilin2a cDNA in pClneo was used to generate profilin2a phospho-mutants using overlapping primers to exchange single serine or tyrosine for aspartic acid. Profilin2a WT and phospho-mimetics were molecularly cloned into pET24a by PCR using primers with added restriction sites for NdeI and XhoI and subsequent restriction digestion. This procedure was done for all phospho-mimetics except for S137D for which site-directed mutagenesis was directly performed on profilin2a cDNA in pET24a vector. The insertions were confirmed by Sanger sequencing. Protein purification was performed according to an earlier protocol. Briefly, protein production in transformed E coli BL21(DE3) was induced with 0.4 mM isopropyl-thio-β-D-galactosidase (IPTG) for 4 hours at 37°C. Cell pellets were resuspended in lysis buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 2 mM DTT supplemented with protease inhibitor). Lysis of cells was performed by incubation of cells with lysozyme, sonication, addition of benzomase, and centrifugation at 21,130 × g. The cleared lysate was put on pre-washed poly-L-proline (PLP)-Sepharose beads (PLP: P2254; Sepharose:
C9142, Sigma-Aldrich, St. Louis, Missouri, USA, prepared according to manufacturer’s instructions) and incubated at 4°C overnight. On the following day, the beads were washed with 10 × to 40 × wash buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1mM EDTA and 2 mM DTT), eluted with wash buffer supplemented with 8 M urea and further purified on a Superose 12 10/300 GL column (17517301, GE Healthcare, Chicago, Illinois, USA). Fractions containing profilin2a were pooled and refolded fast with storage buffer (20 mM Tris-HCl [pH 8.0], 1mM EDTA, 1 mM DTT), and subsequently dialyzed against storage buffer. Preparation of chicken muscle actin was conducted with minor changes as described before.31,32

2.3 | Stability assay

To measure protein thermo-stability in vitro, we used the fast proteolysis assay.33 For each experiment, 5 µM purified profilin2a WT and phospho-mutants were each diluted in assay buffer (50 mM Tris-HCl [pH 8.0], 0.5 mM CaCl2) and mixed with 0.1 g/L thermolysin (P1512, Sigma-Aldrich, St. Louis, Missouri, USA) in assay buffer. Samples of 10 µL were incubated for 1 minute at 4°C, heated to 40-70°C for 1 minute and cooled down to 4°C for 1 minute in a LifeEco Thermal Cycler (Biozym, Hessisch-Oldendorf, Germany). Two samples of profilin2a with or without thermolysin were kept on 4°C and served as controls. Reaction was stopped by adding EDTA and samples were incubated with Laemmli buffer at 95°C for 5 minutes.

2.4 | PIP2-bead coupled binding assay

500 ng of profilin2a WT and phospho-mimetics were each mixed with 12.5 µL phosphatidylinositol 4,5-bisphosphate (PIP2) beads (P-B045a, Echelon Biosciences, Salt Lake City, Utah, USA) in binding/wash buffer (10 mM HEPES [pH 7.4], 0.25% IGEPAL-CA630, 150 mM NaCl) and incubated with rotation at 4°C for 3 hours. Beads were washed with binding/wash buffer with increasing amounts of NaCl (150, 200, 300, 400, and 500 mM). 20 µL of each fraction was incubated with Laemmli buffer at 95°C for 5 minutes.

2.5 | PLP-bead coupled binding assay

1 µM of profilin2a WT and phospho-mutants were each mixed with 50 µL PLP beads in binding/wash buffer (20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM DTT) and incubated with rotation at 4°C overnight. Beads were then washed at RT for 15 minutes with binding/wash buffer with increasing amounts of urea (0, 2, 4, 6, and 8 M). 20 µL of each fraction was incubated with Laemmli buffer at 95°C for 5 minutes.

2.6 | Fluorimetric measurement

The affinity of profilin2a for PLP was determined by monitoring the enhancement of intrinsic tryptophan fluorescence of profilin2a by binding to PLP.34-36 2 µM profilin2a WT and phospho-mimetics were each diluted in assay buffer (10 mM Tris-HCl [pH 7.5], 150 mM KCl, 1 mM EDTA, and 5 mM β-mercaptoethanol) and titrated with increasing amounts of PLP consisting of 10 prolines (0, 1.0, 5.0, 15.6, 31.1, 62.5, 125, 250, 500, 1000, and 2000 µM). Extinction wavelength was set to 295 nm and emission was measured from 310 to 500 nm at a bandwidth of 5 nm in a Jasco FP-8500 spectrofluorometer (Jasco, Pfungstadt, Germany). After normalization to values between 0 and 1, dissociation constants $K_D$ were calculated by plotting the values at 350 nm against the PLP concentration and fitting a sigmoidal curve generated using the Hill equation with the software Origin 2016G (OriginLab Corp., Northampton, Massachusetts, USA).

2.7 | Microscale thermophoresis

Microscale thermophoresis (MST) was used to measure the affinity of profilin2a for G-actin. Experiments were conducted with the Monolith NT.115Pico (Nanotemper, Munich, Germany) in G-buffer (5 mM Tris-HCl [pH 8.0], 0.1 mM CaCl2, 0.2 mM ATP, 0.5 mM DTT) at 30% LED power and 40% (60% for Y133D) MST power. Purified profilin2a WT and phospho-mutants were labeled for detection with Atto 655-maleimide (80661, Sigma-Aldrich, St. Louis, Missouri, USA) which forms a stable thioether linkage with the cysteine residues of profilin2a. Labeling was performed in G-buffer without DTT and at pH 7-7.5 for 30 minutes at RT. Dye was removed from solution by dialysis against G-buffer. Measurements were performed with 40 nM profilin2a and a 1:1 dilution series of actin with a highest concentration at 21.5 µM. The dissociation constant $K_D$ was calculated by plotting data normalized between 0 and 1 against total profilin2a concentration and fitting with the Hill equation.

2.8 | Actin polymerization assay

Chicken muscle actin was labeled with N-(1-Pyrenyljiodoacetamide (pyrene) as described before.31 Pyrene-labeled and non-fluorescent, dark actin in G-buffer (5 mM Tris-HCl [pH 8.0], 0.1 mM CaCl2, 0.2 mM ATP, 0.5 mM DTT) were mixed to a final concentration of 5% labeled actin. The actin-pyrene mix was incubated with 0.2 mM EGTA and 0.05 mM MgCl2 (pH 8)
for at least 10 min on ice and mixed with increasing profilin2a concentrations (0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8, and 10 µM, except for S137D which was only utilized up to 6 µM) diluted in 1 x KMEI buffer (50 mM KCl, 10 mM imidazole [pH 7.0], 1 mM MgCl₂, and 0.1 mM EGTA). Polymerization was induced by the addition of 10xKMEI to a final concentration of 50 mM KCl and 1 mM MgCl₂. Measurements were performed in a 384-well plate using the Synergy4 microplate reader (Bio-Tek, Winooski, Vermont, USA) for 2 hours at an excitation wavelength of 340 nm and emission wavelength of 400 nm. Obtained values were normalized to their minimum with Origin 2016G (OriginLab Corp., Northampton, Massachusetts, USA). Lag phase was defined as the time when 10% of the steady-state fluorescence of actin without profilin2a was reached. The relative extension of lag time was calculated by dividing the lag time measured at each profilin2a concentration by the average lag time at low profilin2a concentrations showing no effect on nucleation. The calculations were performed for WT and each phospho-mimetic independently. Elongation rate at t_{1/2} was determined by fitting a linear regression to the region of half-maximal fluorescence.\(^3\)

### 2.9 Actin spin-down assay

G-actin sequestering capacity was estimated using the Muscle Actin Binding Protein Biochem Kit (BK001, Cytoskeleton, Denver, Colorado, USA) according to the manufacturer's instructions. All steps were performed at room temperature. Briefly, 16 µM G-actin was incubated with 5 µM profilin2a WT or phospho-mutants for 30 minutes before inducing polymerization with actin polymerization buffer for further 30 minutes. Subsequently, the tubes were centrifuged at 150 000 x g for 1.5 hours. The pellets were solubilized in Milli-Q water before adding Laemmli buffer. 2µM BSA or α-actin was used as controls.

### 2.10 Oligomerization assay

Oligomerization of profilin2a WT and phospho-mimetics was investigated with a glutaraldehyde-vapor-induced crosslinking approach.\(^3\) In short, 40 µL of 25% (v/v) glutaraldehyde acidified with 1 µL 5 M HCl was placed on the bottom of a 24-well plate. 10 µL of a 0.25 g/L profilin2a solution in storage buffer (20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM DTT) was put as a drop on a silanized cover slip and turned upside down to seal the well. The plate was incubated at 30°C for 20 minutes, the protein solutions were recovered and incubated with Laemmli buffer at 95°C for 5 minutes. Dimer, trimer, and tetramer formation was judged by the appearance of protein bands with twice and three times of the apparent molecular weight of the monomer.

### 2.11 SDS-PAGE and Western Blot

Proteins were separated by SDS-PAGE and either stained with Coomassie G250 or blotted on a nitrocellulose membrane (Amersham Hybond ECL Nitrocellulose Membrane, GE Healthcare, Chicago, Illinois, USA). For detection, the following antibodies were used: rabbit α-profilin2 (P0101, 1:1000, Sigma-Aldrich, St. Louis, Missouri, USA) as primary antibody and HRP-conjugated α-rabbit secondary antibody (A-11034, 1:4000, Molecular Probes, Invitrogen, Carlsbad, California, USA). Bands were visualized using Immobilon reagent (WBKLS0500, Millipore, Burlington, Massachusetts, USA). Analysis was done using the software LabImage1D (Kapelan, Leipzig, Germany).

### 3 RESULTS

#### 3.1 Generation of human profilin2a phospho-mimetics

Profilin2a is phosphorylated suggesting an important regulatory role in neuronal actin dynamics.\(^17,18\) However, the impact of profilin2a-phosphorylation on functional properties such as the interaction with PIP₂, PLP, and actin remains elusive. First, we employed an in silico strategy to identify putative phospho-sites on the human profilin2a protein. We used the human profilin2a sequence for analysis on the NetPhos 3.1 platform identifying 14 putative phospho-sites (Figure 1A). The algorithms use an artificial neural network approach to predict generic and kinase-specific phosphorylation sites.\(^25,26\) Out of the 14 phospho-sites, seven were selected based on structural criteria such as their relative position to residues which participate in binding\(^11,29,30\) (Figure 1B). All the phospho-sites of interest localize to the surface thus being accessible for kinases. One phospho-site was selected for its exclusive localization to the PLP binding site (Y29) (Figure 1B, Table 1). Two sites are part of the overlapping PLP/PIP₂ region (Y133, S137). S129 is in proximity to all three binding domains. S76 localizes close to the actin/PIP₂ binding sites and S71 is in the center of the actin binding site. Additionally, we chose Y78, which is distant from any binding site (Figure 1B, Table 1). Next, we generated phospho-mimetics exchanging single amino acid residues against aspartic acid by site-directed mutagenesis. We purified the recombinant proteins using poly-L-proline (PLP) coupled beads as
described before. Subsequently, we added size exclusion chromatography further enhancing protein purity (Figure 1C).

**3.2 Reduced thermal stability of S76D and Y78D indicates conformational changes**

The functionality of the phospho-mimetics depends on protein folding. We validated the structural integrity by a fast proteolysis assay. The purified proteins were mixed with the thermostable endoprotease thermolysin and incubated at increasing temperatures from 40 to 70°C. Protein unfolding exposes buried amino acid residues, which become accessible for cleavage by thermolysin. Profilin2a WT underwent thermal denaturation beginning at 47.9°C and reached half-maximal levels at 52.1°C. Only a marginal amount of protein remained stable at 57.1°C, therefore defined as the stability threshold (Figure 2A). Importantly, all of the phospho-mutants displayed a stability threshold far from 37°C (Figure 2B). This demonstrates that the phospho-mimetics are properly folded under physiological conditions. However,
S76D and Y78D displayed a reduced stability threshold compared to WT and the other phospho-mimetics (Figure 2B). Thus, we analyzed temperature-dependent degradation by quantifying protein levels (Figure 2C,D). This confirmed a marked reduction of S76D folding stability, which was even more pronounced for Y78D. Thus, S76D and Y78D are properly folded at physiological temperatures, but phosphorylation at these sites may induce conformational changes.

### 3.3 Y133 and S137 phosphorylation abrogates PIP₂ binding, whereas Y78 is a positive regulator

PIP₂ binding tethers profilin2a to the cell membrane and prevents actin and PLP interactions,⁴,¹¹ thus being a major regulatory mechanism. We tested PIP₂ binding of the phospho-mimetics compared to profilin2a WT using PIP₂-coupled beads (Figure 3). The beads were incubated with the purified proteins which became immobilized via their PIP₂ binding. Next, we washed with NaCl step-wise increasing the concentrations and collected each elution fraction (Figure 3A). The PIP₂–profilin2a binding depends on electrostatic interactions which are sensitive for increasing ion concentrations.⁴⁹,⁵⁰ Thus, Western blot analysis revealed decreasing protein amounts with each subsequent elution step (Figure 3B). Quantification allowed to plot a decay curve and calculate a decay constant (Figure 3C, Table 2). The decay constant is a good estimate for the affinity which is independent of the initial amount of protein attached to the beads. Remarkably, the C-terminal phospho-mimetics Y133D and S137D demonstrated a pronounced decrease in PIP₂ binding. S137 was hardly detectable already at 200 mM NaCl (Figure 3C, Table 2). Conversely, mimicking phosphorylation

| Table 1 | Localization of putative phospho-sites relative to actin, PLP or PIP₂ binding sites |
|-----------------|-----------------|-----------------|-----------------|
| Profilin2a residues | Proximity to binding sites |
| Y29             | PLP             |
| S71             | Actin, PIP₂     |
| S76             | Actin, PIP₂     |
| Y78             | –               |
| S129            | Actin, PIP₂, PLP|
| Y133            | PLP, PIP₂      |
| S137            | PLP, PIP₂      |

**Figure 2** Profilin2a S76D and Y78D show an impaired thermal stability. Temperature-induced unfolding of profilin2a WT and phospho-mimetics was measured by the fast proteolysis assay.⁴³ Samples with and without thermolysin were kept at 4°C as controls. A, Representative Coomassie-stained 15% SDS-PAGE of profilin2a WT after incubation with thermolysin at the indicated temperatures. B, Profilin2a WT and phospho-mimetics were screened for their thermal stability as indicated by the last temperature at which a signal was detectable on SDS-PAGE (mean ± SEM and n = 3 for WT, n = 1 for phospho-mimetics). Dotted lines indicate the temperature range determined for profilin2a WT denaturation. C, Representative Coomassie-stained 15% SDS-PAGEs of profilin2a S76D and Y78D after incubation with thermolysin at the indicated temperatures. D, Signal intensities of profilin2a WT, S76D, and Y78D normalized to intensities of thermolysin bands at each temperature to allow comparison between analyzed proteins. Protein amount detected at 40°C was set to 1 (mean ± SEM, n = 3, multiple unpaired t tests, Holm-Sidak multiple comparison’s test, *P < .05).
at Y78 enhanced PIP2 affinity. Y133D and S137D localize next to C-terminal PIP2 binding site explaining the dramatic reduction in PIP2 interaction (Figure 1B). However, there is no PIP2 domain in proximity to Y78 and the enhanced binding may be induced by a conformational change—as indicated by a reduced thermal stability (Figure 2).

3.4 | C-terminal Y133 and S137 function as PLP switches and Y29 as a fine-tuner

Profilin2a interacts with ubiquitous and neuron-specific PLP proteins. The PLP binding involves a hydrophobic pocket at the C-term. We generated two phospho-mimetics Y133D and S137D found in this domain, while S129D localizes in proximity to PLP (Figure 1B). Y29 does not localize to a central position of this domain, but has been implicated to enable a larger interacting surface for PLP proteins. We tested PLP binding by incubating recombinant proteins with PLP beads. Subsequently, proteins were eluted with urea which disrupts the interaction via denaturation. Similar to the PIP2 binding assay, we used increasing concentrations on the same column. The eluted fractions were quantified by Western blots (Figure 4A,B). Profilin2a WT strongly bound to the beads. Even a urea concentration of 8 M was not able to completely elute the protein. S71D, S76D, and S129D

FIGURE 3  Profilin2a phosphorylation at the C-terminus show decreased PI(4,5)P2 binding. A, Scheme showing experimental setup of the PIP2-bead coupled elution assay. Profilin2a WT and phospho-mimetics were bound to PIP2 beads and eluted with step-wise increasing concentrations of NaCl. Fractions were collected for further study. B, Representative Western blots of profilin2a WT and phospho-mimetic fractions eluted with increasing NaCl concentrations as indicated. C, Densitometric analysis of profilin2a Western blots. Values were normalized to the band with the highest intensity. Curves were fitted using a one-phase decay equation (mean ± SEM, n = 3, two-way ANOVA, Dunnett’s multiple comparison’s test, **P < .01, ****P < .0001 (applicable for values obtained with buffer + 200 mM NaCl)).
showed a similar behavior which indicates that these sites are not involved in PLP binding. Y78D had a tendency for a reduced affinity at lower urea concentrations which resulted in less eluted protein at higher concentrations. Y29D, Y133D, and S137D showed a pronounced shift of eluted proteins to lower urea concentrations. We validated these findings employing fluorescence spectroscopy. The intrinsic tryptophan fluorescence of profilin2a increased with PLP titration which can be used to calculate the dissociation constant $K_D$ (Figure 4C). Confirming the results of the elution experiment, Y29D, Y133D, and S137D displayed a lower affinity as indicated by higher $K_D$ values. Thereby, Y29D was less affected. This indicates that this site allows fine-tuning while Y133 and S137 exert a switch-like mechanism. Y78D had the same $K_D$ compared to the WT. This difference to the elution experiment may be explained by a higher sensitivity for unfolding agents such as urea. Indeed, Y78D was also more sensitive to thermal denaturation (Figure 2D).

### 3.5 Multiple phospho-sites modulate actin binding and nucleation, whereas elongation is increased by S76 phosphorylation

Profilin2a directly regulates actin in neurons: G-actin-profilin2a binding is a critical step for the dynamic polymerization and depolymerization of F-actin. Thus, we studied the binding ability of WT and mutants for G-actin using microscale thermophoresis (MST) with labeled proteins (Figure 5A). MST uses the directed movements of biomolecules within a microscopic temperature gradient—a

| Table 2 | Decay rate constants obtained by fitting with one-phase decay equation |
|---------|---------------------------------------------------------------|
| Profilin2a | Rate constant (1/s) |
| WT | 3.64 ± 0.22 |
| Y29D | 3.62 ± 0.19 |
| S71D | 3.55 ± 0.37 |
| S76D | 3.14 ± 0.07 |
| Y78D | 2.10 ± 0.33 |
| S129D | 3.46 ± 0.22 |
| Y133D | 6.24 ± 0.74 |
| S137D | 17.05 ± 11.45 |

**Figure 4** Profilin2a Y133D and S137D show a pronounced decrease in PLP binding capacity. A, Profilin2a WT and phospho-mimetics were bound to PLP beads and eluted with step-wise increasing concentrations of urea. Representative Western blots of profilin2a WT and phospho-mimetic fractions eluted with urea concentrations as indicated. B, Densitometric analysis of profilin2a Western blots. Values were normalized to the band with the highest intensity (mean ± SEM, n = 3, one-way ANOVA, Dunnett’s multiple comparison’s test, **$P < 0.01$, ****$P < .0001$). C, Dissociation constant $K_D$ of profilin2a-PLP interaction was estimated by measuring the intrinsic fluorescence enhancement of profilin2a titrating with increasing concentrations of PLP. Analysis was performed for profilin2a WT and selected phospho-mimetics which demonstrated a difference in their affinity for PLP in (B) compared to WT.
basic property of biomolecules in aqueous solutions which changes with complex formation.\textsuperscript{44} By increasing the concentration of G-actin, we determined equilibrium dissociation constants ($K_D$) for the interaction by fitting to Hill equations (Table 3). Residue S71 is part of the actin binding site and phosphorylation completely disrupts the ability of profilin2a to bind to G-actin (Figure 5A, Table 3). In contrast, all other phospho-mimetics were able to

**FIGURE 5** Profilin2a S71D and S76D show impaired actin binding capacities. A, Dissociation constants $K_D$ were determined using microscale thermophoresis (MST) with 40 nM labeled profilin2a WT and phospho-mimetics and increasing concentrations of actin. B, Actin pyrene assay to measure actin polymerization in the presence of increasing concentrations of profilin2a. Graph shows polymerization of 2 µM G-actin with 5% pyrene-labeled protein in the presence of 4 µM profilin2a WT and phospho-mimetics over a time frame of 2 hours. Vertical line indicates the 10% threshold defining lag time. C, Extension of the lag time in the presence of 4 µM profilin2a WT and phospho-mimetics relative to the average lag times at concentrations not showing an effect on nucleation. (mean ± SEM, n = 3, one-way ANOVA, Dunnett’s multiple comparison’s test, **$P < .01$, ****$P < .0001$ compared to WT). D, Elongation rate of actin polymerization in fluorescence units (FU) per second in the presence of 4 µM profilin2a at half-maximal fluorescence. (mean ± SEM, n = 2-3, one-way ANOVA, Dunnett’s multiple comparison’s test, *$P < .05$).
TABLE 3  Fitting of the MST data with the Hill equation obtaining the $K_D$ and the Hill coefficient

| Profilin2a | MST   |
|-----------|-------|
|           | $K_D$ (µM) | Hill coefficient |
| WT        | 0.15 ± 0.05 | 1.00 ± 0.20 |
| Y29D      | 0.18 ± 0.03 | 1.51 ± 0.20 |
| S71D      | –      | –             |
| S76D      | 0.31 ± 0.06 | 0.82 ± 0.09 |
| Y78D      | 0.65 ± 0.08 | 0.94 ± 0.07 |
| S129D     | 0.95 ± 0.21 | 0.96 ± 0.09 |
| Y133D     | 0.30 ± 0.02 | 1.12 ± 0.05 |
| S137D     | 0.36 ± 0.04 | 1.03 ± 0.12 |

efficiently bind to G-actin. Y29D showed the same binding behavior as the WT reflected in similar $K_D$ values. The distant localization of the mutated residue from the actin binding site has no influence on the binding property (Figure 1B). S129D and S76D, on the other hand, are located close to the actin binding domain and accordingly exhibited a reduced affinity for G-actin which was more pronounced for S129D. Interestingly, Y78D, Y133D, and S137D displayed also a reduced binding affinity to G-actin although they are located close to the actin binding domain. The Hill coefficient ($n_H$) gives information about the cooperativity of an interaction between proteins and ligands.45 The fitting procedure yielded in all cases Hill coefficients close to one (Table 3) demonstrating non-cooperative binding of the phospho-mimetics to G-actin including WT.

Next, we evaluated the functional consequences of profilin2a-phosphorylation on the polymerization of actin. Therefore, we used the actin-pyrene assay to follow in real time the kinetics of actin polymerization (Figure 5B). We initiated the polymerization reaction by enhancing the ionic strength of the solution through the addition of salts either without any profilin2a, or containing the WT or the phospho-mimetics. The polymerization reaction can be separated into a lag phase, an elongation phase, and a steady state. The lag phase is determined by a rate-limiting step of actin nucleation and seed formation. We determined the lag times from the delay in polymerization in the actin-only control (Figure 5C). Profilin sequesters actin monomers thereby inhibiting nucleation. Thus, the lag time extends in the presence of profilin and reaches a maximal value at saturating profilin concentrations.2 Since we measured actin polymerization at different profilin concentrations, we were able to calculate the effects of the phospho-mimetics on the lag time (Figure 5C, S1). S71D had even at high concentrations no effect on the lag time at subsequent phases of polymerization which is in accordance with the MST data, which revealed its inability to bind to G-actin (Figure 5A-C, Table 3). In the case of S76D, S129D, and S137D, the lag phase was reduced compared to the WT. This is consistent with weak actin-binding affinity (Figure 5C). Notably, Y78D, Y133D, and Y29D displayed a more complex behavior on the polymerization. Y29D inhibited nucleation less efficient than the WT, although the binding to actin monomers was comparable (Figure 5C, Table 3). Moreover, Y78D and Y133D showed a WT-like inhibition of actin nucleation, but displayed decreased G-actin binding especially for Y78D (Figure 5C, Table 3). These data indicate that the influence of the phospho-mimetics on actin dynamics cannot simply be explained by the effect of G-actin binding assuming that additional steps of the polymerization reaction are involved. In line with that, there is another layer of complexity since profilins form oligomers with yet largely undefined functional consequences. Crosslinking experiments with mild conditions revealed substantial impact of profilin phosphorylation on oligomerization with Y78D displaying enhanced multimerization (Figure S2).

The elongation phase is characterized by the rapid attachment of G-actin to the barbed end of the filament. Both the WT and the majority of the phospho-mimetics had no influence on the elongation rate (Figure 5D). Under these defined experimental conditions, the elongation process appeared to be independent of the G-actin binding properties of profilin and its sequestration ability. However, S76D accelerated actin polymerization demonstrating an activating effect of this mutation. Interestingly, the phospho-mimetic S76D showed the strongest interaction with F-actin in spin-down assays compared to the other mutants providing a mechanistic base for the increased actin polymerization rate (Figure S3).

4  | DISCUSSION

Neuronal profilin2a is a major regulator of neurotransmitter homeostasis at the synapse.46 This is mediated by the interaction with synaptic PLP proteins such as dynamin1 or piccolo involved in endocytosis and synaptic-vesicle recycling, as well as the direct binding to actin and lipids like PIP$_2$.3-5,24 Neurotransmitter release and recycling is tightly regulated and relies on fine-tuned actin dynamics. Not surprisingly, profilin2a is phosphorylated at multiple sites17,18 indicating that profilin-phosphorylation might be critical in this process. However, the exact consequences of profilin2a-phosphorylation have not been studied so far.

Here, we investigated the impact of specific profilin2a phospho-sites on its interaction with PLP, PIP$_2$, actin monomers as well as on actin polymerization. All of these properties were influenced by profilin2a-phosphorylation (Table 4). This suggests an important regulatory mechanism for neuronal cells which will be addressed in future studies. Phosphorylation at S71, S76, and S129 interfered only with actin while other properties remained unchanged.
S71 directly localizes to the actin binding pocket and serves as a molecular switch: phosphorylation completely abrogates any interference with the actin cytoskeleton. However, PLP binding was not affected. PLP binding of profilin2a has a biological function independent of the regulation of actin dynamics. Profilin2a competes for dynamin-1 binding with other endocytic proteins via its PLP domain. Thus, profilin2a can still interfere with synaptic endocytosis when switched-off by S71-phosphorylation. S129-phosphorylation results in similar changes but there is a remaining influence on actin dynamics suggesting a role in fine-tuning of profilin2a activity. While phosphorylation at S71 and S129 dramatically changed actin binding with an unaffected PLP domain, Y29 did the opposite. PLP binding was slightly reduced with an almost absent effect on actin. In a cellular system, this would allow fine-tuning of endocytosis.

Interestingly, profilin2a-phosphorylation at S76 enhanced the elongation rate of polymerizing actin and strengthened the interaction with filamentous actin. The influence of profilin on elongation is still under debate. However, the interaction of profilin with filamentous actin may be critical for that. Cross-linking experiments shed light on this mechanism. Profilin which was closely cross-linked with actin not allowing any conformational change blocked actin polymerization. This was different from a flexibly cross-linked actin profilin which allowed a conformational change. Here, polymerization was not disturbed. This suggests that there is no need to fully dissociate profilin from the barbed end. The removal of the steric hindrance is sufficiently independent of profilin’s binding to F-actin. Interestingly, profilin forms dimers and multimers with a yet unknown role in actin dynamics. Thus, it is possible that profilin interacts with other profilin molecules at the barbed end facilitating attachment of the next profilin-actin. An enhanced elongation rate combined with a stronger F-actin binding, as observed for S76D, would fit into this model.

Phosphorylation at S137 affected all binding properties. PIP2, PLP, and actin binding were diminished resulting in a minor effect on actin nucleation compared to WT (Table 4). A reduced interaction with all binding partners could be mimicked by a profilin2a knockdown. In a cellular context, a profilin2a knockdown reduced the F-actin content and caused neurite sprouting. Moreover, an enhanced neurotransmitter release at the pre-synapse, and destabilized spines at the post-synapse was observed. S137 is conserved among profilin isoforms and the Rho kinase phosphorylates profilin1 at this amino acid residue. Phosphorylation at Y133 had similar consequences on the binding properties compared to S137 (Table 4). However, the reduced G-actin binding had no influence on the nucleation compared to the WT. A possible explanation would be other regulatory mechanisms at the growing barbed end during nucleation which need to be resolved in future studies.

Y78D was the only phospho-mimetic with an enhanced PIP2 binding. At the same time, actin binding was reduced. Profilin bound to PIP2 at the membrane is unable to bind to actin. This represents a profilin storage pool ready to be released upon PLC stimulation. Our results are consistent with these findings. Y78-phosphorylation enhanced the PIP2 and reduced the actin binding. Thus, it may be the underlying mechanism behind profilin sequestration at the membrane.

Dysregulation of profilins has been linked to neurodegenerative diseases. Eight different mutations of profilin1 have been associated with familiar forms of the motoneuron disease ALS. Mutated sites localize close to the actin or PLP binding sites comparable to the phospho-sites in profilin2a (Table S1). Similar to our profilin2a mutant S129D, the profilin1 G118V displayed a marked decrease in G-actin binding. Interestingly, mice harboring the G118V profilin1 mutation developed an ALS-like pathophysiology with a tendency for an altered F/G-actin ratio before disease onset becoming significant at onset and late-symptomatic stages.

TABLE 4 Summary of site-specific effects of profilin2a phospho-mimetics

|        | Y29D | S71D | S76D | Y78D | S129D | Y133D | S137D |
|--------|------|------|------|------|------|------|------|
| Thermal stability | --- | --- | ↓ | ↓↓ | --- | --- | --- |
| PIP2 binding | --- | --- | --- | ↑ | --- | --- | ↓ |
| PLP binding | ↓ | --- | --- | --- | --- | --- | --- |
| Actin binding | --- | n.d. | ↓ | ↓↓ | ↓↓ | --- | --- |
| Extension of lag time | ↓ | n.d. | ↓ | --- | ↓ | --- | ↓ |
| Elongation rate | --- | --- | ↑ | --- | --- | --- | --- |
| Oligomerization | --- | ↓ | ↓ | ↑ | ↑ | ↓ | --- |

Note: n.d. = not determined, --- = WT-like, ↓ = reduced compared to WT, ↑ = enhanced compared to WT.
This demonstrates the importance of profilin-linked actin dynamics for the pathology of motoneuron-diseases. Profilin1 is ubiquitously expressed. This indicates that motoneurons are remarkably vulnerable for dysregulations downstream of profilin. Not surprisingly, changes at the neuronal profilin2a are involved in the pathogenesis of another motoneuron disease, spinal muscular atrophy (SMA). SMA is a monogenic disease caused by the loss of the SMN-protein which directly interacts with profilin2a. In SMA, profilin2a becomes hyper-phosphorylated at yet undefined phospho-sites emphasizing the disease-relevance of this process. The question why profilin1 is a risk factor for ALS while SMA-pathology involves profilin2a is still open. A main difference between both proteins is their binding to PLP: compared to profilin1, profilin2a has a higher affinity to PLP proteins such as SMN. However, profilin1 could also be involved in SMA-pathology. Vice versa, profilin2a may play a role in ALS since it potentially forms heterodimers with profilin1. Our study uncovers several sites with regulatory features that alter dimerization as well as the interaction properties of profilin2a with actin, PLP and PIP2. The resulting functional consequences of phosphorylation may thus decipher the underlying molecular mechanism of motoneuron diseases.

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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS
LM Walter and P. Claus designed research; LM Walter, P. Franz, and R. Lindner performed research; LM Walter, P. Franz, N. Hensel, R. Lindner, G. Tsiavaliaris, and P. Claus analyzed data; LM Walter, N. Hensel, G. Tsiavaliaris, and P. Claus wrote the manuscript.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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