Fascin is Enriched in Dendritic Protrusions and Regulates Synaptic Plasticity

[1,2,3] James Q Zheng, [1] Shuristeen Joubert, [1] Carlos Gonzalez-Islas, [4] Yuki Ogawa, [1] Abhishek Poddar, [1] Arjolyn B Penas, [1] Enyu Liu, [1] Peter Wenner and [1] Kenneth R Myers

1. Department of Cell Biology, Emory University School of Medicine, Atlanta, GA 30322

2. Department of Neurology, Emory University School of Medicine, Atlanta, GA 30322

3. Center for Neurodegenerative Disease, Emory University School of Medicine, Atlanta, GA 30322

4. Department of Biological Sciences, McCausland College of Arts and Sciences, University of South Carolina, Columbia, SC 29208

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Correspondence: James Zheng, PhD, Department of Cell Biology, Emory University, 615 Michael Street, Atlanta, Georgia 30322, USA. Email: james.zheng@emory.edu;

Kenneth R Myers, PhD, Department of Cell Biology, Emory University, 615 Michael Street, Atlanta, GA 30322, USA. Email: kennethmyers@emory.edu.

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Abstract

The Fascin family of actin-bundling proteins organizes actin filaments (F-actin) into tightly packed bundles that drive dynamic membrane protrusions such as filopodia. In neurons, fascin has been thought to primarily function in axons, as previous studies reported its absence from dendritic filopodia and spines. Here, we demonstrate that fascin is both present and functionally important in dendritic compartments. Using optimized immunocytochemistry and CRISPR-based endogenous tagging of fascin1 in cultured hippocampal neurons, we show that fascin localizes to developing dendritic filopodia and is enriched in mature dendritic spines. Super-resolution imaging further reveals that fascin is organized into discrete nanoscale foci within spine heads, but not the spine neck. Finally, we show that CRISPR-mediated knockout of fascin1 in mature hippocampal neurons impairs synaptic potentiation, without affecting baseline excitatory synaptic transmission. Together, our findings uncover a previously overlooked aspect of actin organization in dendritic spines and establish fascin as a critical regulator of postsynaptic plasticity.

Summary Statement

The actin bundling protein fascin localizes to dendritic filopodia and spines, where it regulates activity-dependent synaptic plasticity.

Introduction

Chemical synapses are specialized sites of connection that enable neuronal communication underlying sensorimotor behaviors, learning, memory, and cognition. Synapses are composed of pre- and post-synaptic structures specialized for neurotransmitter release and reception, respectively. These structures are highly plastic, undergoing both functional and structural modifications in response to activity (Bin Ibrahim et al., 2022; Ge et al., 2010; Malenka, 1994). This includes alterations in postsynaptic surface neurotransmitter receptors and presynaptic neurotransmitter release probability (Anggono and Huganir, 2012; Benke and Traynelis, 2019; Bosch and Hayashi, 2012; Citri and Malenka, 2008; Monday and Castillo, 2017). Synaptic plasticity represents the cellular basis for learning and memory (Martin et al., 2000; Takeuchi et al., 2014), and its disruption contributes to a wide range of neurological disorders (Lima Caldeira et al., 2019; Nishiyama, 2019; Stefen et al., 2016; Walker and Herskowitz, 2021).

Most excitatory synapses in the vertebrate brain reside on dendritic spines, which are tiny actin-rich protrusions that concentrate postsynaptic components for effective excitatory synaptic transmission (Chidambaram et al., 2019; Hotulainen and Hoogenraad, 2010; Nimchinsky et al., 2002). Spines undergo structural changes in shape and size during development and synaptic plasticity that are strongly associated with learning, aging, and neurodegenerative diseases (Lima Caldeira et al., 2019; Nishiyama, 2019; Stefen et al., 2016; Walker and Herskowitz, 2021). Dendritic filopodia, which act as spine and synaptic precursors, are thin actin-rich structures that sample the neuropil for axonal partners before stabilizing upon contact (Lohmann and

Bonhoeffer, 2008; Spence et al., 2016). Both dendritic filopodia and spines depend upon the actin cytoskeleton for their formation, remodeling, and maintenance (Bucher et al., 2020; Hotulainen and Hoogenraad, 2010; Lei et al., 2016; Okabe, 2020). Unlike conventional filopodia, which contain tightly bundled unipolar arrays of parallel actin filaments, dendritic filopodia and thin spine necks contain branched and straight actin filaments of mixed polarity that do not form tight bundles (Hotulainen et al., 2009; Korobova and Svitkina, 2010). These networks are organized and remodeled by a large number of actin binding proteins (ABPs) (Chhabra and Higgs, 2007; Pollard et al., 2000; Pollard and Borisy, 2003; Revenu et al., 2004). During structural long-term potentiation (sLTP), distinct sets of ABPs sequentially reorganize, stabilize, and consolidate the actin network in individual spines (Bosch et al., 2014). Actin-bundling proteins are essential for the reorganization and stabilization steps during sLTP, although the specific ABPs and their precise roles in these processes remain unclear (Bosch et al., 2014).

Fascin is a highly conserved actin-bundling protein best known for generating tightly packed filamentous actin (F-actin) bundles in dynamic membrane protrusions such as filopodia and invadopodia (Adams, 2004; Jansen et al., 2011; Krey et al., 2016; Li et al., 2010; Vignjevic et al., 2006). In neurons, fascin has been mostly studied in developing axons, where it localizes to growth cone filopodia and contributes to axon elongation and guidance (Cohan et al., 2001; Hardin et al., 2025; Sasaki et al., 1996). However, fascin is reportedly absent from dendritic filopodia and spines (Korobova and Svitkina, 2010; Parker et al., 2023), leading to the prevailing view that fascin plays no role in postsynaptic actin organization or plasticity. In this study, we challenge this

model by showing that fascin is present and functionally important in dendritic compartments. Using a combination of confocal microscopy, super-resolution imaging, and CRISPR/Cas9 gene editing, we show that fascin is enriched in dendritic filopodia and mature spines, where it forms discrete nanoscale foci within spine heads. Functionally, we demonstrate that CRISPR-based knockout of fascin1 in mature hippocampal neurons impairs activity-dependent synaptic potentiation without affecting baseline neurotransmission. These findings reveal an unexpected role for fascin in the modulation of the dendritic actin cytoskeleton and establish fascin as a critical regulator of synaptic plasticity.

Materials and Methods

Cell culture

Dissociated primary hippocampal neuron cultures were prepared as previously described (Gu et al., 2010). Briefly, 18.5-day-old embryos were collected from timed-pregnant Sprague Dawley rats (Charles River Laboratories), and hippocampi were dissected in ice-cold Hank's Balanced Salt Solution (HBSS). Hippocampi were pooled together, trypsinized for 12 minutes, briefly incubated in 20% fetal bovine serum, and plated on 25 mm acid-washed glass coverslips coated with 100 µg/ml poly-L-lysine (P2636; Sigma-Aldrich) at a density of approximately 300,000 cells per 35 mm dish. Hippocampal neurons were cultured in Neurobasal medium (21103049; Gibco) supplemented with B-27 (17504044; Gibco), penicillin/streptomycin (30002CI; ThermoFisher), and GlutaMax (35050061; Gibco), and fed ½ volume of fresh medium every 7 days post-plating. Animal care and use was conducted following National Institutes of Health guidelines, and procedures were approved by the Institutional Animal Care and Use Committee at Emory University.

Low-density hippocampal cultures were prepared using a feeder layer of cortical neurons according to the previously published method (Lu et al., 2016). Here, hippocampal neurons were plated on poly-L-lysine-coated 25 mm glass coverslips at a density of 25,000 cells per well in a 6-well culture plate. In a separate 6-well culture plate, several small paraffin wax dots ~2 mm in diameter and ~1 mm in height were planted on the bottom of each well, serving as spacers. After coating the bottom of each well with 100 µg/ml poly-L-lysine, cortical neurons from the same embryonic brains were plated at a density of 400,000 cells per well. After 24 h in culture, the coverslips with

low-density hippocampal neurons were removed from their plate and placed in the 6-well plate of cortical neurons with the hippocampal neurons facing down towards the cortical neurons. Weekly ½ volume medium changes were carried out as described above.

The mouse neuroblastoma cell line, Cath.-a-differentiated (CAD) (Qi et al., 1997), was maintained in DMEM/F12 (10-092-CV; Corning) supplemented with 8% fetal bovine serum (Atlanta Biologicals) and 1% Penicillin/Streptomycin (Invitrogen). To obtain CAD cells with elaborated lamellipodia and filopodia, we re-suspended the cells and plated them on laminin-coated (20 µg/ml) glass coverslips for 2-3 hours at 37°C before fixation/immunofluorescence or live cell imaging (Pollitt et al., 2020; Vitriol et al., 2015).

Transient transfection and AAV-based transduction

GFP-tagged proteins were transiently expressed in CAD cells using X-tremeGENE HP DNA transfection reagent (Pollitt et al., 2020; Vitriol et al., 2015).

To endogenously tag fascin1 in cultured hippocampal neurons, we utilized the Homology-Independent Universal Genome Engineering (HiUGE) method (Gao et al., 2019; Ogawa and Rasband, 2021) and adeno-associated virus (AAV) to tag the N-terminus of Fascin1 in cultured rat hippocampal neurons. Custom single-stranded DNA oligonucleotides encoding guide RNAs (gRNAs) targeting the N-terminus (5'-TTGGCGGTCATGGTGGCGGA) of Fascin1 were synthesized, annealed, and subcloned into the pAAV-pHiUGE-smFP-V5 donor-ORF0 plasmid (Addgene #200380) after SapI restriction enzyme digestion (Ogawa et al., 2025). β-actin was endogenously tagged with EGFP using pAAV-pHITI-Actb-GFP as described (Ogawa et al., 2025) to identify and visualize sparsely distributed edited neurons for high- and super-resolution

imaging of fascin in dendritic spines. To knockout fascin1, we employed a previously described (Ogawa et al., 2023) AAV-mediated multiplexed gRNA system. We inserted the following 3 gRNAs targeting Fascin1 into pAAV-3xgRNA-KO (Addgene #240310): TCTACCGCGATCTCGTCGGC, GCGACCTTCGCAGTCGCGAA, and GCACCCACACGGGCAAGTAC. As the control, we used the pAAV-3xRNA-KO-template which encodes three non-targeting gRNAs: CTGTCTTCATCATGGCCGAC, GTTCGCATTATCCGAACCAT, TAAGCGTCGCAAGAAGACGG. To express Cas9, we used a modified version of pAAV-pMecp2-SpCas9-spA (Addgene #60957) lacking the HA-tag. The following high-titer AAV particles were produced with the PHP.S serotype by VectorBuilder (Chicago, IL): pAAV-pHiUGE-smFP-V5-Fascin1, pAAV-pHITI-Actb-GFP, pAAV-3xgRNA-KO template, pAAV-3xgRNA-KO-Fascin1, and pAAV-pMecp2-SpCas9. For transduction of cultured neurons, we added AAV-Mecp2-SpCas9 (MOI = 50,000) together with either pHiUGE-smFP-V5-Fascin1, HITI-Actb-GFP, 3xgRNA-KO template, or 3xgRNA-KO-Fascin1 (MOI = 50,000). We applied AAV particles to DIV4 neurons for 48 hours before washout and maintained the cells until DIV21 or later for imaging and electrophysiology.

Electrophysiology

Whole-cell voltage clamp recordings were obtained from DIV21-23 rat hippocampal neurons expressing bright smFP-HA using an AxoPatch 200B amplifier (Molecular Devices), controlled by pClamp 10.1 software (Molecular Devices), low pass filtered at 5 KHz on-line and digitized at 20 KHz. Tight seals (>2 GΩ) were obtained using thin-walled boro-silicate glass microelectrodes pulled to obtain resistances between 7 and 10 MΩ. The intracellular patch solution contained the following (in mM):

potassium-gluconate 125, KCl 10, Tris-phosphocreatine 5, Mg-ATP 3, Na_2-GTP 1.5, HEPES 10, $MgSO_4$ 2, $CaCl_2$ 0.1, EGTA 0.5. The pH was adjusted to 7.4 with KOH. Osmolarity was measured between 280-300 mOsm. The recording chamber was mounted on an Axiovert 100 Microscope (Karl Zeiss Microscopes) and continuously perfused (5 ml/min) with oxygenated Artificial Cerebral-Spinal Fluid (ACSF) recording solution containing the following (in mM): NaCl 126, KCl 3, NaH2PO4 1, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10 and D-glucose 25. The pH was adjusted to 7.4 with NaOH. Miniature excitatory postsynaptic currents (mEPSCs) were isolated using 1 µM TTX (R&D Systems) and 5 µM Gabazine (R&D Systems) in ACSF to block action potentials and GABAergic mIPSCs, respectively. Membrane potential was held at -70 mV and recordings were performed at 27 □C. For chemically induced LTP, recording solution was switched to oxygenated LTP-induction solution containing (in mM): tetraethylammonium chloride (TEA-Cl) 25, $CaCl_2$ 5, $MgCl_2$ 0.1, NaCl 97, KCl 3, NaH_2PO_4 1, HEPES 10 and D-glucose 25 for 10 min. Series resistance during recordings varied from 15 to 20 MΩ and was not compensated. Recordings were terminated whenever significant increases in series resistance (> 20%) occurred. Analysis of mEPSCs was performed blind to condition with MiniAnalysis software (Synaptosoft) using a threshold of 5 pA for mEPSC amplitude. Estimation statistics were used. 5000 bootstrap samples were taken; the confidence interval was bias-corrected and accelerated. The p values reported are the likelihoods of observing the effect sizes if the null hypothesis of zero difference is true. For each permutation p value, 5000 reshuffles of the control and test labels were performed (Ho et al., 2019).

<u>Antibodies and immunofluorescent labeling</u>

The following antibodies were used: rabbit polyclonal anti-GFP (Invitrogen, #A11122; 1:1000), mouse monoclonal anti-α-tubulin (clone DM1A, Sigma, #MABT205; 1:1000), mouse monoclonal anti-fascin (clone 55K2, Millipore Sigma, #MAP3582; 1:1000), rabbit monoclonal anti-fascin (Abcam, #EP5902, KO validated; 1:500), and sheep polyclonal anti-fascin (R&D Systems, #AF7745, KO validated; 1:1000). Mouse anti-fascin antibody was generated using purified full-length human fascin, and recognizes fascin from mouse, rat, and human. Rabbit anti-fascin antibody was generated using amino acids 200-400 from recombinant human fascin and recognizes fascin from human and mouse (predicted to recognize rat). Sheep anti-fascin antibody was generated using full-length human fascin1 and it detects human, rat, and mouse fascin. Triple channel immunofluorescent staining of cultured CAD cells using these three anti-fascin antibodies was found to label the same F-actin structures, with quantitative analysis showing a very high degree of colocalization as indicated by Pearson Correlation Coefficients, Manders' Colocalization Coefficients, and Costes' P-values (Supplemental figure S1 A-B).

For confocal imaging, Alexa Fluor-conjugated (AF488-, AF546-, and/or AF647) secondary antibodies raised in Donkey were diluted (1:500) in phosphate-buffered saline (PBS) containing 2% donkey serum for 1.5 hours. For STED imaging, long Stokes-shifted Abberior STAR 460L-conjugated goat anti-rabbit, STAR ORANGE-conjugated goat anti-mouse, and STAR RED-conjugated donkey anti-sheep secondary antibodies were used. Due to the cross-reactivity of goat and sheep IgGs, we employed a two-step labeling approach. Cells labeled with three primary antibodies (mouse, rabbit, and sheep) were first incubated with STAR RED-conjugated donkey anti-sheep

antibodies for 1.5 hours at room temperature, followed by three washes with PBS, PBS-0.2% Tween-20, and PBS, respectively. Cells were then incubated with both STAR 460L and STAR ORANGE-conjugated goat antibodies for 1.5 hours at room temperature, followed by three washes, and mounted in ProLong Gold antifade mount (Thermo Fisher, #P36930) for over 2 days before STED imaging.

The optimal fixation method for immunofluorescent labeling of fascin is the use of 100% cold methanol. Briefly, cultured hippocampal neurons were rapidly fixed with 100% cold anhydrous methanol (Sigma 322415) for 10 min at -20°C, followed immediately by rehydration in PBS containing 0.1% Triton X-100, and three additional washes in PBS. Cells were blocked in PBS with 2% normal donkey serum (Sigma D9663) for 1 hour at room temperature. Neurons were incubated in a humidified chamber overnight at 4°C with primary antibodies diluted in PBS with 2% donkey serum. Cells were washed with PBS, PBS-0.05% Tween 20, and PBS, followed by incubation with the mixture of secondary antibodies for 1.5 h at room temperature. After three washes with PBS, PBS-Tween20, and PBS, the coverslips were mounted on glass slides using ProLong Gold antifade.

Microscopy and imaging

Laser scanning confocal microscopy was performed on a Nikon C2 confocal unit with four laser lines (405 nm, 488 nm, 561 nm, and 610 nm), three detectors, and either 10X/NA0.5 or 60X/NA1.4 Plan Apo objectives (Nikon Instruments Inc, Melville, New York, USA). Confocal image acquisition was done using Nikon Elements software. Stimulated Emission Depletion (STED) super-resolution imaging was performed using an Abberior Facility Line STED system (Abberior Instruments GmbH, Göttingen,

Germany) housed on an Olympus IX83 inverted microscope (Evident, Tokyo, Japan) with a 60X/NA1.4 oil immersion objective (UPLXAPO, Olympus, Japan). With the use of long Stokes-shifted Abberior STAR 460L together with STAR ORANGE and STAR Red, a single 594 nm pulsed STED depletion laser was used for simultaneous triple channel STED imaging using 485 nm, 560 nm, and 640 nm excitation lasers. Image acquisition was done using Inspector software (Abberior, Germany), and post-acquisition image enhancement and quantification were done using FIJI ImageJ software (NIH, Bethesda, MD).

Western blots

Rat hippocampal neurons were lysed directly in 1× Laemmli sample buffer at indicated timepoints, then boiled for 5 min, and vortexed for 5 min. Equal volumes of cell lysates were loaded on mini-Protean 12% Tris-glycine acrylamide gels (#4561043, Bio-Rad) and transferred to nitrocellulose. Membranes were blocked with 5% milk in PBS-Tween (0.2%) for 1 h, then incubated overnight at 4°C with rabbit anti-Neuronal Class III beta-tubulin polyclonal (#PRB-435P, Covance; 1:2000) and mouse anti-fascin (1:5000). Membranes were washed 3x in PBS-Tween then incubated in anti-rabbit-Alexa Fluor568– (Invitrogen) and anti-mouse-IRDye800-conjugated (LiCor) secondary antibodies for 1 h at room temperature. Membranes were washed 3x in PBS-Tween and then visualized using a ChemiDoc MP imaging system (Bio-Rad, Hercules, CA).

Results and discussion

A unique requirement for fixation methods to preserve the spatial localization of fascin.

Fascin is the primary actin-bundling protein in filopodia and other cellular protrusions, yet its reported subcellular localization by immunocytochemistry has been inconsistent depending on how cells are fixed. Because the localization of an actin-binding protein fundamentally shapes how its function is interpreted, resolving these discrepancies is critical for understanding fascin's function in cells, particularly in neurons where actin-based structures are diverse and functionally specialized. Previous work noted that fascin localization to F-actin bundles can be revealed with cold methanol fixation but not aldehyde-based fixation, though the mechanistic basis for this difference remained unexplained (Ross et al., 2000; Yamakita et al., 2011; Yamashiro et al., 1998). To systematically evaluate how fixation conditions affect the spatial localization of fascin in cells, we examined mouse neuroblastoma CAD cells, which form prominent lamellipodia and filopodia when seeded on laminin substrates (Skruber et al., 2020; Vitriol et al., 2013). Consistent with previous findings (Hardin et al., 2025; Vignjevic et al., 2006), cold 100% methanol (-20°C) preserved fascin enrichment in F-actin-rich lamellipodia and filopodia, as evidenced by using multiple independent anti-fascin antibodies (Figure 1A and Figure S1). However, cold methanol fixation disrupts the binding of phalloidin to F-actin (Figure 1A), as reported previously (Burkel et al., 2007; Svoboda, 1992). Conversely, fixation with either 2% glutaraldehyde or 4% paraformaldehyde (PFA) resulted in robust phalloidin staining but weak or no fascin signals in actin-rich structures, rendering fascin cytosolic (Figure 1A and Figure S2A). Similarly, fixation with 50% cold methanol (Korobova and Svitkina, 2010) failed to

preserve fascin localization and resulted in a substantial loss of fascin signals associated with F-actin in membrane protrusions (Figure S2B). In both aldehyde- and 50% methanol-fixed CAD cells, only weak fascin signals were observed in association with thick F-actin bundles in membrane protrusions (arrows in Figure 1A and Figure S2). Similar to CAD cells, aldehyde-based fixation also diminished fascin signals associated with F-actin in nerve growth cones in culture (Figure S3). These results demonstrate that cold 100% methanol, not aldehyde-based cross-linking, is required to preserve and reveal the association of fascin with distinct F-actin structures. The substantial loss of fascin signals associated with F-actin upon aldehyde or 50% methanol fixation could lead to underestimation of fascin enrichment in specific cellular compartments, producing incorrect interpretations about the molecular composition of actin-based structures.

Aldehyde-based cross-linking can impair antibody detection by masking specific epitopes (Metz et al., 2004; Otali et al., 2009; Scalia et al., 2017). To test whether this mechanism accounts for the loss of fascin signal, we expressed exogenous GFP-fascin and stained cells with both anti-GFP and anti-fascin antibodies. Aldehyde-based fixations, but not 100% methanol fixation, eliminated the colocalization of GFP-fascin with F-actin bundles (Figure 1B). Critically, this loss of colocalization was observed with both antibodies, including an anti-GFP antibody known to reliably detect GFP in PFA-fixed cells (Myers et al., 2022; Myers et al., 2020), arguing against a simple epitope-masking explanation. We therefore considered an alternative mechanism that aldehyde fixation physically displaces fascin from actin filaments before cross-linking can occur. To test this directly, we performed real-time imaging of GFP-fascin and mCherry-actin

during the fixation process and found that GFP-fascin dissociates from F-actin almost immediately upon addition of 2% PFA, whereas most mCherry-actin signals persist (Figure 1C). This observation is consistent with the known kinetic properties of fascin–actin interactions: fascin exhibits rapid and dynamic binding behavior when bundling actin filaments, with a kinetic off-rate of ~0.12 s^{-1} (Aratyn et al., 2007; Winkelman et al., 2016). In contrast, formaldehyde fixation proceeds through a relatively slow two-step process: an initial, rapid Schiff base formation (seconds) followed by a much slower cross-linking step requiring minutes or longer to form stable bonds (Hoffman et al., 2015). This temporal mismatch means that formaldehyde cannot cross-link fascin to actin filaments before fascin dissociates. Instead, formaldehyde likely inactivates fascin molecules while they are transiently unbound, trapping them in a cytosolic state, a model strongly supported by the live-imaging data (Figure 1C).

Together, these findings establish that rapid fixation using cold methanol (or cold acetone; data not shown) is essential for accurately visualizing fascin localization in cells, and they provide a mechanistic explanation for long-standing discrepancies in fascin localization across studies. More broadly, these results carry important implications for interpreting published data: studies that relied on aldehyde fixation or reduced methanol concentrations, to conclude that fascin is absent from specific actin-based structures, such as dendritic spines, should be re-evaluated, as the previously reported absence of fascin may reflect a fixation artifact rather than a genuine biological observation.

<u>Fascin is enriched in dendritic protrusions.</u>

During synaptic development, dendritic processes elaborate dendritic filopodia

that are considered precursors of postsynaptic dendritic spines (Lohmann and Bonhoeffer, 2008; Spence et al., 2016). A previous study using 50% methanol fixation concluded that fascin is absent from dendritic filopodia, unlike conventional filopodia and axonal growth cone filopodia (Korobova and Svitkina, 2010). Given that 50% methanol fixation fails to preserve fascin association with F-actin (Figure S2B), we re-examined fascin localization in dendritic compartments using cold 100% methanol fixation. Since fascin is highly enriched in axonal compartments (Hardin et al., 2025), we employed very low-density hippocampal cultures (10,000 cells/35mm dish) to examine dendritic fascin distribution and minimize interference from axonal signals. At DIV11, when filopodia are the predominant dendritic protrusion type, we observed robust enrichment of fascin in dendritic filopodia, colocalizing with F-actin (Figure S4A, arrowheads). We also observed fascin enrichment in dendritic protrusions at DIV13 (Figure S4B), a stage characterized by a mix of dendritic filopodia (Figure S4B, arrowheads) and immature dendritic spines that contact axons and exhibit slightly enlarged heads (Figure S4B, arrows). Spine morphology became much more apparent by DIV18, as protrusions contacting axons exhibited clearly enlarged heads enriched with fascin (Figure S4C, arrows). Fascin signals in dendritic protrusions are relatively weaker than in axonal growth cones (GC, Figure S4B), but their presence in both filopodia and immature spines is clearly evident.

By DIV25, dendritic spines have become predominant in low-density hippocampal cultures, where fascin can clearly be detected and appears enriched in spines (Figure 2A). To confirm this observation, we examined fascin localization in mature dendritic spines in high-density hippocampal cultures (250,000 cells/35mm

dish). We took advantage of sparse CRISPR/Cas9-based GFP tagging of endogenous β-actin (GFP-endo-β-actin) in high-density cultures. GFP signals were used to create a mask that removed surrounding signals from neighboring non-transduced cells, enabling clearer examination of fascin enrichment in individual dendritic spines. Consistent with our observations in low-density cultures, fascin is enriched in mature dendritic spines in high-density hippocampal cultures (Figure 2B). Quantitative analysis of the spine-to-shaft ratio confirmed elevated levels of fascin in spines relative to the neighboring dendritic shaft in both low-density and high-density cultures (Figure 2C). Together, our data demonstrate that fascin is not only present but also enriched in dendritic filopodia and spines, which directly contradicts the prior conclusion that fascin is absent from dendritic protrusions (Korobova and Svitkina, 2010) and suggests a potential role for fascin in postsynaptic structure and function.

Fascin in spines forms nanoscale foci

Having established that fascin is enriched in dendritic spines, we next sought to investigate whether fascin exhibits a nanoscale organization in spines. Here, we utilized CRISPR-based gene editing to fluorescently tag endogenous fascin1 at its N-terminus with spaghetti monster fluorescent protein with V5 epitopes (smFP-V5). Consistent with our immunostaining data, smFP-V5-endo-fascin1 signals are enriched in dendritic spines, as well as in the growth cone with filopodia (Figure 3A). Tagged endo-fascin1 signals appear to be mostly enriched in the spine heads and exhibit a punctate pattern (Figure 3A, yellow arrows), suggesting a sub-spine organization of fascin. To investigate the nanoscale distribution of fascin in spines, we next performed super-resolution STED imaging. Here, high-density hippocampal cultures were subjected to GFP-tagging of

endogenous β-actin *via* AAVs, fixed with cold 100% methanol at DIV25, and triple-labeled for GFP (for GFP-endo-β-actin), bassoon (for presynaptic active zones), and fascin. While fascin signals are clearly enriched in actin-rich dendritic spines under confocal microscopy, STED imaging reveals that fascin is distributed as discrete foci throughout the actin-rich spine head, but rarely in the spine neck (Figure 3B-C, Figure S5A). We find that individual fascin foci in spines ranged in size from 40 to 160 nm, with a mean of 97 nm (Figure 3D). This punctate pattern of fascin signals in spines contrasts sharply with the continuous fascin distribution along F-actin bundles in lamellipodia and axonal growth cones (Figure S5B-C), indicating that these nanoscale foci are not a result of fixation or immunolabeling artifacts. Rather, this organization is consistent with the absence of tight F-actin bundles in spines, suggesting that fascin may serve a distinct, bundle-independent function in regulating the dendritic spine F-actin network.

Fascin is essential for synaptic plasticity.

To test the function of fascin in dendritic spines, we utilized AAV-based CRISPR/Cas9-mediated knockout (KO) of fascin1 in cultured hippocampal neurons. Neurons were infected at DIV4 with AAVs expressing spCas9 and either non-targeting control gRNAs (3xgRNA-KO template) or three gRNAs targeting the coding region of FSCN1 (3xgRNA-KO-Fascin1). Fascin was effectively knocked out, as confirmed by immunostaining and western blotting (Figure S6A-D). At DIV25, HA-positive neurons (indicating gRNA-expressing cells) displayed essentially no fascin fluorescence (Figure S6A). Western blotting revealed a relatively late onset of fascin KO (~12 days post-infection) in cultured hippocampal neurons, with residual fascin levels likely attributable to a small fraction of non-transduced cells (Figure S6C-D). This delayed timeline is

consistent with a recent report showing continued editing of post-mitotic cells for at least 16 days after viral sgRNA and Cas9 infection (Ramadoss et al., 2025). We also found that fascin expression gradually increases over the first three weeks of development in culture (Figure S6E), consistent with previous reports (De Arcangelis et al., 2004; Zhang et al., 2008).

We found no differences in either dendritic spine density or morphology between the control (3xgRNA-KO template) and KO (3xgRNA-KO-Fascin1) neurons (Figure S7A-D). Likewise, whole-cell patch-clamp recordings revealed no difference in the baseline frequency or amplitude of miniature excitatory postsynaptic currents (mEPSCs) between the control and KO neurons (Figure 4A-D). This suggests that Fascin1 is not required for the development of mature dendritic spines and synapses under basal conditions. To test whether fascin is required for synaptic plasticity, we used a well-established chemical LTP (cLTP) protocol in which tetraethylammonium (TEA) is applied to block potassium channels (Gu et al., 2010; Hille, 1967; Huang and Malenka, 1993). Consistent with previous findings, brief exposure to 25 mM TEA resulted in increased spine size (Figure S7E) and produced robust potentiation of mEPSC frequency and amplitude in control neurons (Figure 4B-C, 4E). In contrast, fascin KO neurons showed no increase in spine size (Figure S7F). Interestingly, rather than increasing, both mEPSC frequency and amplitude decreased after TEA treatment (Figure 4B-C, 4F). These results identify fascin as a key actin regulator required for activity-dependent synaptic remodeling.

The actin cytoskeleton is highly enriched in postsynaptic spines where it not only supports the distinct spine shapes but also provides a scaffold for the spatiotemporal

organization of postsynaptic receptors and signaling components (MacGillavry et al., 2013; Okabe, 2020). Importantly, postsynaptic remodeling during learning and memory depends on highly regulated actin dynamics, and its disruption is strongly associated with neurological conditions (Bosch and Hayashi, 2012; Cingolani and Goda, 2008; Hotulainen and Hoogenraad, 2010). Despite tremendous progress in understanding actin-based cellular activities, we still lack a detailed picture of actin organization and dynamic regulation in synaptic compartments. In this study, we establish fascin as a key actin regulator of postsynaptic structure and function, revising the prevailing view that fascin is neither present nor functionally relevant in dendritic compartments. These findings are consistent with recent studies identifying fascin1 as a target for spine formation and memory consolidation (Shivkumar et al., 2025; Yang et al., 2025).

How fascin regulates the spine F-actin network remains unknown. Our STED imaging reveals that fascin forms discrete nanoscale foci in spine heads rather than the continuous distribution seen along F-actin bundles in filopodia and lamellipodia. Determining which F-actin structures fascin engages within these foci would be an important next step. Such structural characterization is typically achieved through electron microscopy approaches, particularly platinum replica electron microscopy, as employed previously to examine actin architecture in spines (Korobova and Svitkina, 2010). However, these EM approaches require strong chemical fixation and detergent extraction steps that, as we demonstrate here, displace fascin from F-actin. This technical limitation likely explains why fascin was not detected in dendritic protrusions in earlier ultrastructural studies. Future advances in cryo-electron microscopy of neuronal

samples may ultimately enable visualization of fascin-actin interactions in spines under near-native conditions.

Given the lack of tight actin bundles in spine heads, it is plausible that fascin adopts a distinct organization within the mostly branched F-actin network to function in a complementary, but non-overlapping, manner from other actin-bundling proteins in spines (Chazeau and Giannone, 2016; Winkelman et al., 2016). For example, α-actinin-mediated crosslinking of actin filaments is important for proper spine development and maintenance, as well as for linking actin to the postsynaptic density (Hlushchenko et al., 2018; Wyszynski et al., 1997). However, unlike α-actinin, which localizes throughout dendritic spines (Hodges et al., 2014; Wyszynski et al., 1998), the appearance of fascin in discrete nanoscale foci specifically within spine heads (Figure 3B) suggests a role in fine-tuning actin dynamics within the densely branched actin network of the spine head. Recent studies indicate that fascin binding to F-actin strongly impairs cofilin-mediated actin turnover, even without forming thick actin filament bundles (Chikireddy et al., 2024), raising the possibility that fascin could regulate actin turnover within the branched F-actin network of dendritic spines. In this model, a small number of fascin molecules engaging neighboring filaments within the branched spine network could locally modulate cofilin-mediated regulation of actin dynamics, which is important not only for control of spine structural remodeling but also for the regulation of synaptic receptor surface insertion (Gu et al., 2010). Recent evidence in non-neuronal contexts also supports a role for fascin in the regulation of branched actin networks (Chikireddy et al., 2024; Wubshet et al., 2021). Finally, it is notable that TEA-cLTP produced a decrease, rather than simply a failure of potentiation, in both mEPSC frequency and

amplitude in fascin KO neurons. One possibility is that the activity-dependent calcium influx triggered by cLTP induction drives cofilin-dependent actin remodeling in spines that, in the absence of fascin-mediated stabilization, results in a net loss of synaptic efficacy rather than potentiation. Additionally, because changes in mEPSC frequency can reflect presynaptic alterations, and fascin is known to be present in developing axons (Hardin et al., 2025), it will be important to determine whether fascin also contributes to actin organization in mature presynaptic terminals. Future studies are needed to elucidate the precise mechanism by which fascin engages with F-actin in postsynaptic compartments, whether it also functions presynaptically, and how these roles contribute to synaptic plasticity.

Figure Legends

Figure 1. Methanol fixation is required to capture the cellular localization of fascin. (**A**) Representative immunofluorescent images of CAD cells fixed with 100% cold methanol (top row) or 2% glutaraldehyde (bottom row). Cells were stained with mouse (M) anti-fascin, rabbit (R) anti-fascin, and phalloidin-AlexaFluor546. Regions enclosed by dashed rectangles are shown at a higher magnification on the right, highlighting the actin-rich lamellipodia and filopodia. Arrows highlight weak fascin signals remaining after aldehyde fixation. (**B**) Representative images of CAD cells expressing GFP-fascin for approximately 24 hours and fixed with either 100% cold methanol (left panels) or 4% PFA (right panels). Cells were stained with mouse (M) anti-fascin and rabbit anti-GFP. (**C**) Sequential images showing live CAD cells co-expressing GFP-fascin and mCherry-γ-actin, before and after fixation with 2% PFA (added at 0" seconds). Time shown in seconds (total duration 70 seconds). Scale bars = 10 µm.

Figure 2. Fascin is enriched in mature dendritic spines. (**A**) Representative images of low-density cultured hippocampal neurons at DIV28 fixed with 100% cold methanol and labeled for endogenous fascin (cyan) and actin (magenta). Left scale bar = 10 µm, right scale bar = 5 µm. (**B**) Representative image of a neuron from high-density hippocampal culture expressing GFP-tagged endogenous β-actin, fixed with 100% cold methanol at DIV28, and labeled for fascin (cyan) and GFP (magenta). GFP-β-actin signal was used as mask to isolate individual neuron from neighboring cells. Right, high magnification panels of boxed regions from image on left. Left scale bar = 10 µm, right scale bars = 2 µm. (**C**) Quantification of fascin enrichment in dendritic spines. Graphs show the ratio of fascin or β-actin fluorescence intensity in spine heads compared to the

intensity of an equal-sized area of the neighboring dendritic shaft. *n* = fields of view, representing >200 spines from low-density cultures and >500 spines from high density cultures, from 3 independent cultures. Error bars are ± SEM.

Figure 3. Fascin is enriched in dendritic spine heads as discrete nanoscale foci. (**A**) Representative images of hippocampal neurons at DIV21 with endogenous fascin1 tagged by CRISPR-mediated knock-in of smFP-V5 (cyan). The left panel shows a portion of the dendritic arbor of a neuron expressing smFP-V5-fascin1 (cyan) within the high-density hippocampal culture immunofluorescently labeled for actin (magenta). Fascin signals are present in dendritic arbors and enriched in dendritic spines, as well as an axonal growth cone (GC). The regions enclosed by dashed rectangles are shown in a high magnification on the right, in which fascin signals are represented using a cyan-hot look-up table. Yellow arrows indicate spines with punctate fascin distribution within the spine heads, whereas white arrows indicate small spines with fascin in their heads. Left scale bar = 10 µm, right scale bars = 2 µm. (**B**) Representative confocal (top row) and STED (bottom row) images of high-density hippocampal cultures expressing GFP-tagged endogenous β-actin, fixed at DIV25 with 100% cold methanol, and triple stained for fascin (cyan), β-actin (magenta), and bassoon (yellow). GFP-β-actin signal was used as mask to isolate individual neurons from neighboring cells. Fascin appears as discrete foci within actin-rich spine heads (arrows), but not in spine necks. A portion of the dendrite (dashed rectangle) is shown on the right, in a higher magnification with three channels merged. Scale bars = 1 µm. (**C**) High magnification images of individual spines in (B) showing punctate fascin distribution (spines outlined in blue). Scale bar = 1 µm. (**D**) Histogram showing width of individual fascin foci in spine heads, assigned

within 20 nm bins. Gray bar shows accumulated frequency of foci width. A total of 6 STED images and more than 30 spines were analyzed.

Figure 4. Fascin is required for synaptic potentiation in mature hippocampal neurons. (**A**) Representative images showing the identification of fluorescent neurons for patch-clamp recording. (**B**) Representative sample traces of mEPSCs from whole-cell patch-clamp recordings of control (template) and fascin KO (3gRNA) before and after application of 25 mM TEA. (**C**) Average mEPSC waveforms before (black) and after (gray) TEA application in control (top) and fascin1 KO (bottom) neurons. (**D-F**) Gardner-Altman estimation plots showing the differences in mEPSC frequency (top) and amplitude (bottom) between the control (N=23) and fascin KO (N=22) groups. (D) The baseline synaptic transmission was not affected by fascin KO, as the unpaired mean differences for the frequency and amplitude are 0.387 Hz (95% confidence interval (CI), -0.13, 1.15) and 0.694 pA (95% CI, -1.25, 2.77). P values of the two-sided permutation *t*-test on the frequency and amplitude are 0.25 and 0.52, respectively. (E) TEA-cLTP significantly increased the frequency and amplitude of synaptic transmission in the control group. The unpaired mean differences before and after TEA treatment are 1.06 Hz (95% CI, 0.503, 1.6) and 2.55 pA (95% CI, 0.639, 4.61). (F) TEA-cLTP did not increase, but decreased, the frequency and amplitude of synaptic transmission in the fascin KO group. The unpaired mean differences before and after TEA treatment are -0.935 Hz (95% CI, -1.59, -0.466) and -2.08 pA (95% CI, -3.96, -0.478). ***: P<0.001, * P<0.05 (two-sided permutation *t*-test).

Supplemental Figures

Figure S1. Fascin localization after methanol fixation is detected with multiple antibodies. (**A**) Representative images of CAD cells fixed with 100% cold methanol (−20 °C) and stained with three independent anti-fascin antibodies (raised in mouse (M), rabbit (R), and sheep(S)). (**B**) Left, magnified image from the boxed region of (A), showing essentially the same fascin enrichment pattern in lamellipodia and filopodia using three independent antibodies. Right, fluorescence intensity profiles corresponding to the dotted white line from image on the left. (**C**) Quantification of colocalization, analyzed for each pairing of anti-fascin antibodies.

Figure S2. Loss of F-actin associated fascin signals in CAD cells fixed with paraformaldehyde or 50% methanol. (**A**) Representative immunofluorescent images of CAD cells fixed with 4% paraformaldehyde (PFA) stained with mouse (M) anti-fascin (cyan), fluorescent phalloidin (yellow), and rabbit (R) anti-fascin (magenta). Arrows indicate weak fascin fluorescence associated with a few thick F-actin bundles in filopodial protrusions. (**B**) Fixation with 50% methanol compromises fascin labeling, compared to fixation with 100% methanol. Representative images of CAD cells fixed with either 100% or 50% cold methanol (−20°C) and stained with mouse (M) anti-fascin and rabbit (R) anti-fascin antibodies. Arrows indicate weak fascin immunofluorescent signals associated with a few thick F-actin protrusions in 50% methanol-fixed cells.

Figure S3. Fascin signals in nerve growth cones with different fixation methods. (**A**) Representative images of nerve growth cones from DIV2 hippocampal neurons in culture fixed by 100% methanol and labeled for fascin and β-actin. A clear overlap between fascin and β-actin signals can be seen in the growth cone. (**B**) Representative

images of neuronal growth cones from cultured DIV2 hippocampal neurons fixed by 4% PFA. While β-actin signals highlight the F-actin bundles in the growth cones, fascin appears to be diffuse and does not highlight F-actin.

Figure S4. Fascin is enriched in dendritic filopodia and immature spine-like protrusions during neuronal development. (**A**) Representative images of low-density cultured hippocampal neurons at DIV11 fixed with 100% cold methanol and labeled for endogenous fascin (cyan) and actin (magenta). The inset in right panel is a linescan showing correlation of fascin and actin levels across multiple filopodia from highlighted region. Arrowheads highlight filopodia with high fascin enrichment. Scale bar = 10 µm. (**B**) Representative images of low-density cultured hippocampal neurons at DIV13 fixed and stained as above. Arrows highlight nascent spines with slightly enlarged heads and fascin enrichment that are in contact with axons. GC: axonal growth cone. Scale bar = 10 µm. (**C**) Representative images of DIV18 neurons fixed and stained as above. Panels on right represent high magnification of corresponding boxed regions from left. Arrows highlight spines with enlarged heads, and arrowheads highlight filopodia. Left scale bar = 10 µm, right scale bars = 2 µm.

Figure S5. STED imaging of fascin. (**A**) Representative confocal image of a DIV25 hippocampal neuron expressing GFP-tagged endogenous β-actin, fixed with 100% cold methanol, and labeled with mouse anti-Bassoon, rabbit anti-GFP, and sheep anti-fascin antibodies. Right, STED images of boxed regions from merged image on left, showing fascin foci in dendritic spine heads. (**B**) Representative confocal and STED images of CAD cell lamellipodia, labeled for endogenous fascin (cyan) and actin (magenta), showing continuous fascin staining pattern along F-actin bundles. (**C**) Representative

STED images of growth cones from primary hippocampal neurons, labeled for endogenous fascin (cyan) and actin (magenta), showing continuous fascin staining pattern along F-actin bundles.

Figure S6. Fascin1 KO validation in hippocampal neurons. (**A**) Representative immunofluorescence images of methanol-fixed DIV25 hippocampal neurons that were infected on DIV5 with AAV to express Cas9 and either control (3xgRNA-KO template) or fascin KO (3xgRNA-KO-Fascin1), both of which co-express smFP-HA. Methanol-fixed cells were immunostained for HA to identify transduced cells (magenta) and evaluate fascin levels (cyan). Fascin immunoreactivity is absent in HA-positive fascin KO neurons. (**B**) Violin and box plot showing the loss of fascin fluorescence intensity in KO cells relative to control neurons. N = number of cells analyzed from 3 separate cultures. (**C**) Representative western blots showing the levels of fascin and tubulin (used as a loading control) at various times post AAV infection (starting at DIV 5). (**D**) Analysis of western blots, showing the effective KO of fascin 12 days post-AAV infection. For each timepoint, samples were normalized to tubulin loading control, and fascin levels in control and KO cells were presented relative to eGFP-expressing neurons. n = 3 separate neuronal cultures. (**E**) Quantification of fascin levels during primary neuron development. Fascin levels were normalized to tubulin and presented relative to the initial DIV6 timepoint. Error bars are ± SEM.

Figure S7. Dendritic spines at baseline and after TEA-induced chemical LTP. (**A**) Representative images of dendritic spines from control (3xgRNA-KO template) or fascin KO (3xgRNA-KO-Fascin1) high-density hippocampal neurons at DIV25-28. Co-expressed smFP is used to visualize spine morphology. Scale bar = 2 µm. (**B-D**) Plots

show no differences in spine density, length, or width between control and fascin KO cells under basal conditions (not significant by unpaired Student's *t*-test). Error bars are ± SEM. (**E**) Representative fluorescent images of dendritic regions from control and fascin KO neurons showing the spine size changes before and 20 min after TEA treatment. Arrows indicate the spines whose sizes increased at 20 min after TEA treatment. (**F**) The plot shows the changes in spine size 20 min after TEA treatment compared to that before TEA. The integrated fluorescence intensity of >150 individual spines per condition, before and after TEA treatment, from 6 independent experiments were quantified. P-value for unpaired student's *t*-test is 0.0361. *P<0.05 (Student *t*-test). Error bars are ± SEM.

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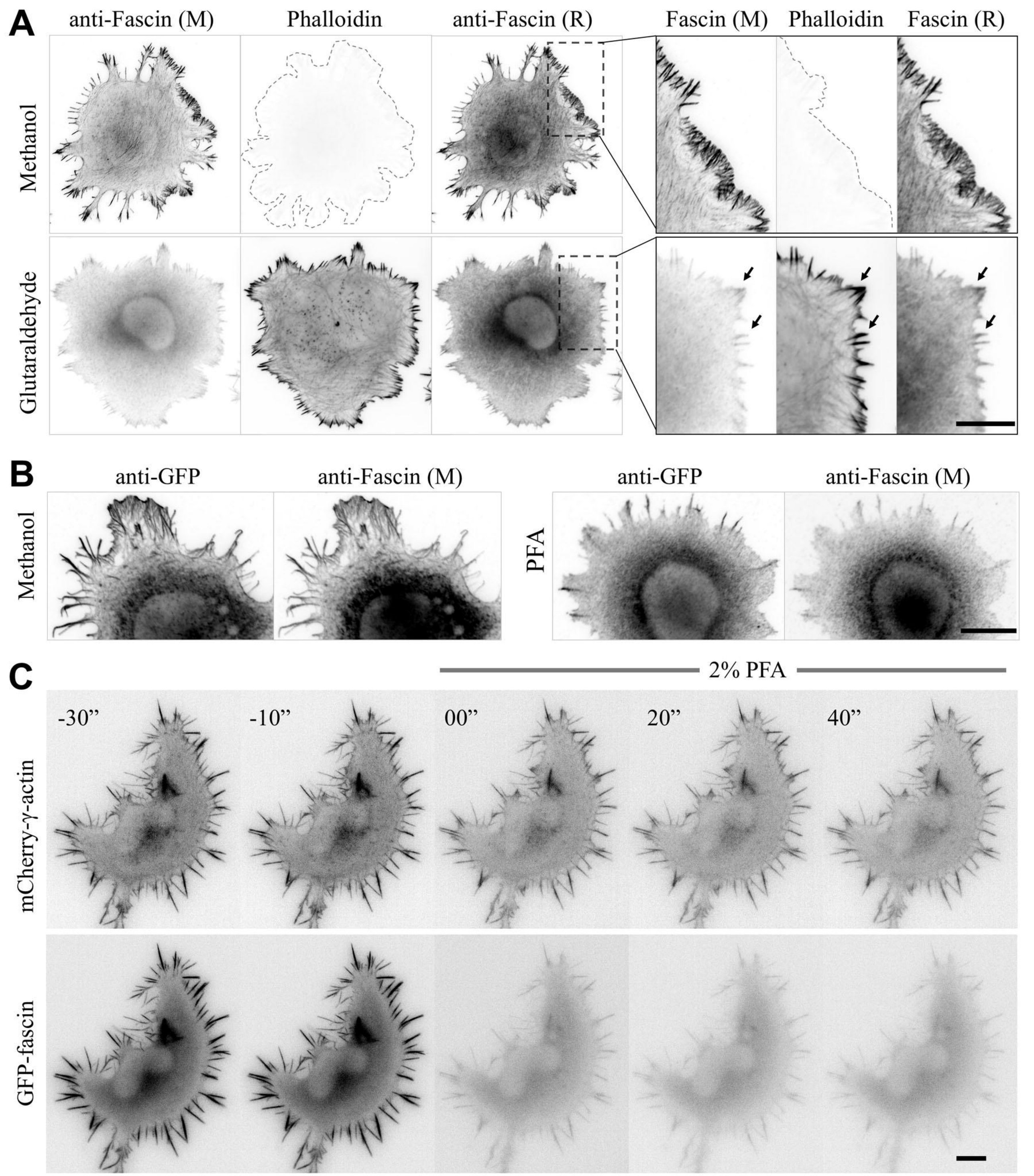
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anti-Fascin (M)
Phalloidin
anti-Fascin (R)
Fascin (M)
Phalloidin
Fascin (R)
Methanol
Glutaraldehyde
B
anti-GFP
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anti-GFP
anti-Fascin (M)
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C
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-30”
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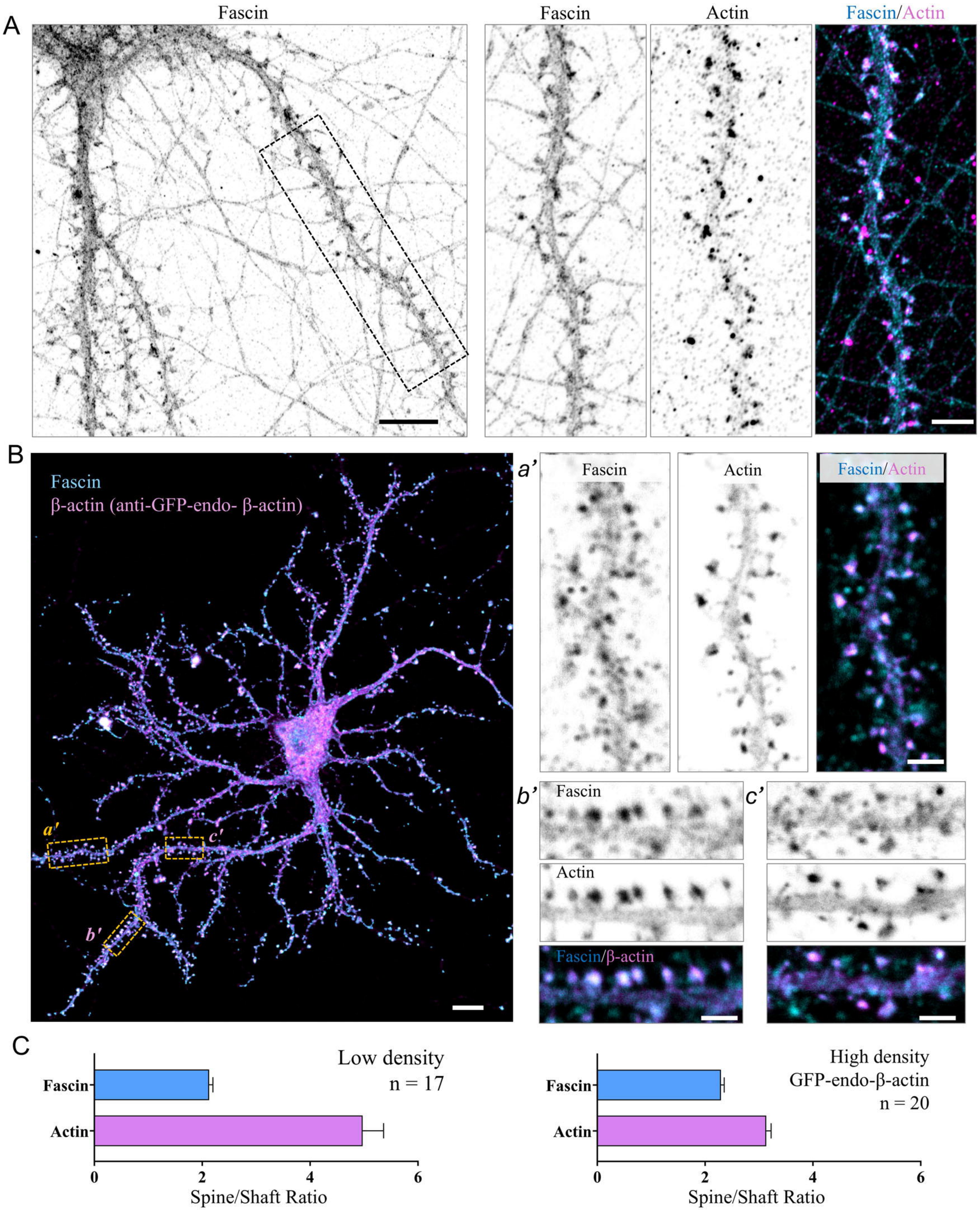
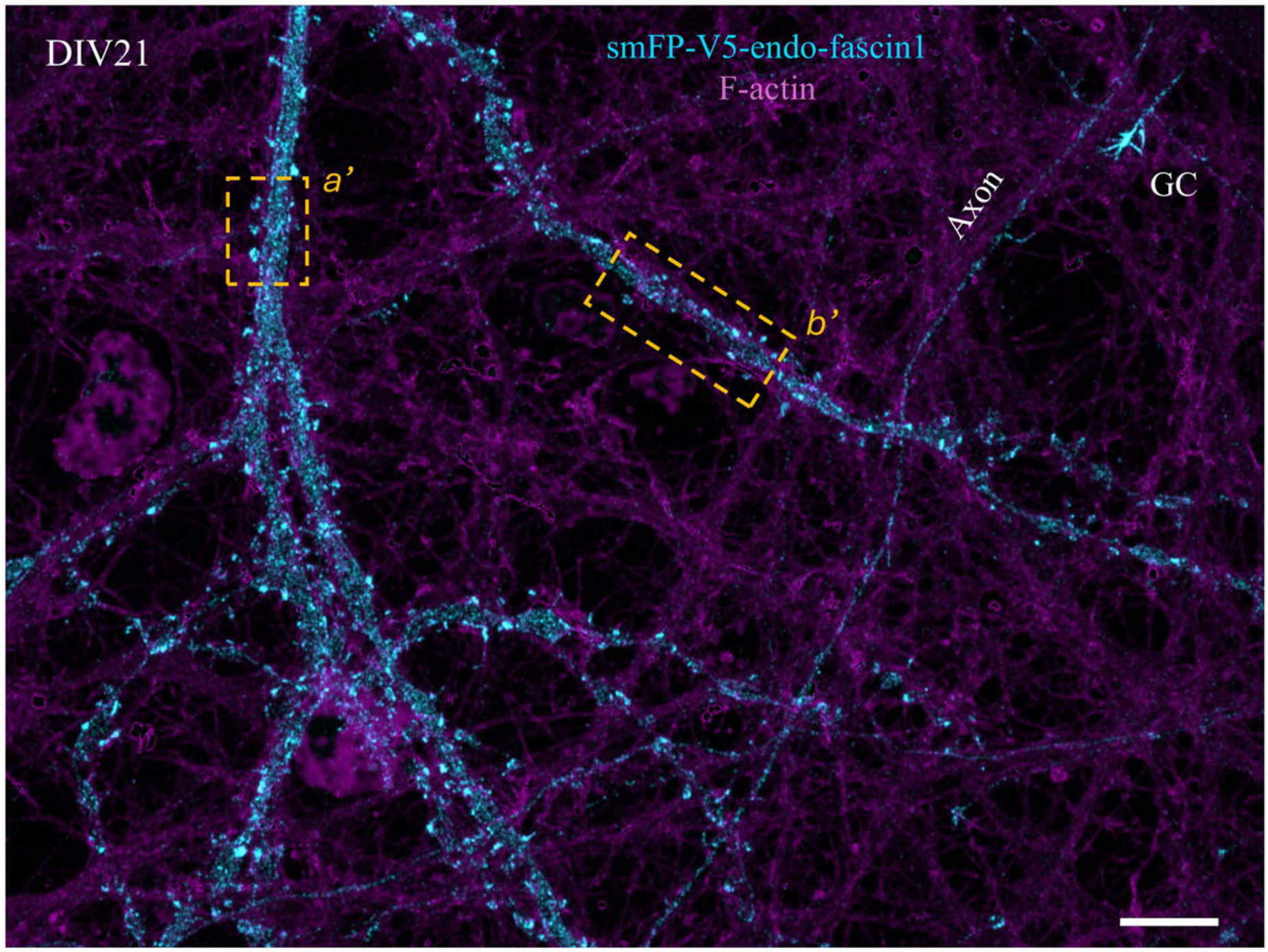
GFP-fascin


A
Fascin
Fascin
Actin
Fascin/Actin
B
Fascin
β-actin (anti-GFP-endo- β-actin)
a'
b'
c'
a'
Fascin
Actin
Fascin/Actin
b'
Fascin
Actin
Fascin/β-actin
c'
C
Low density
n = 17
Fascin
Actin
0
2
4
6
Spine/Shaft Ratio
High density
GFP-endo-β-actin
n = 20
Fascin
Actin
0
2
4
6
Spine/Shaft Ratio

A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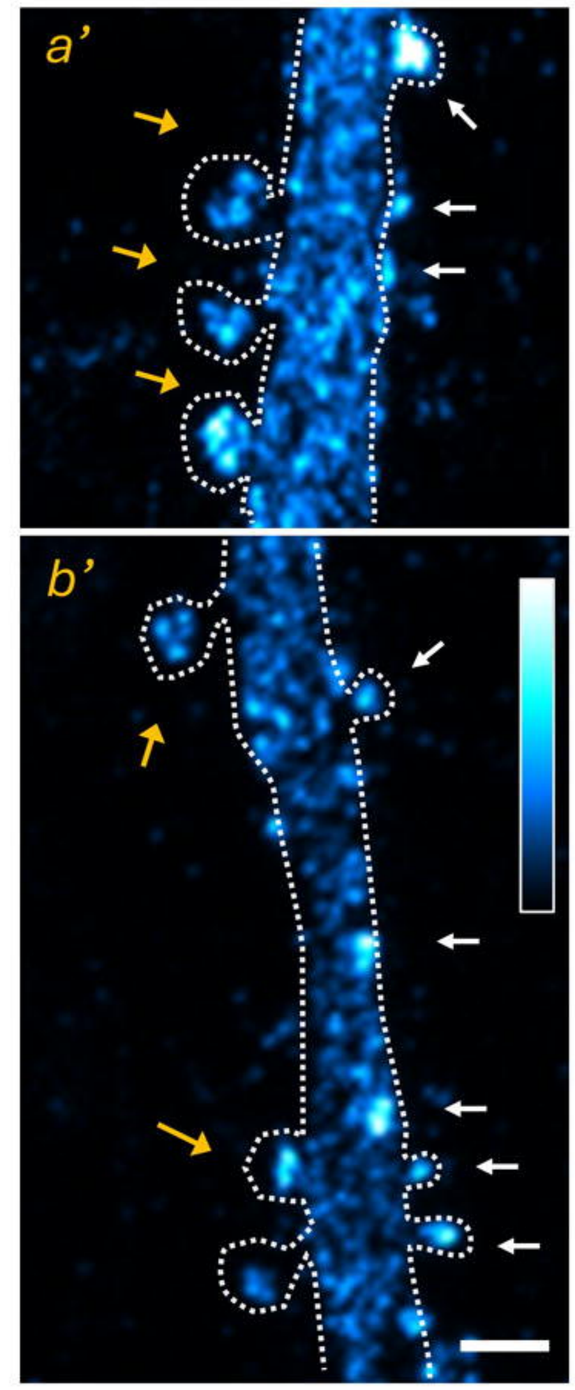
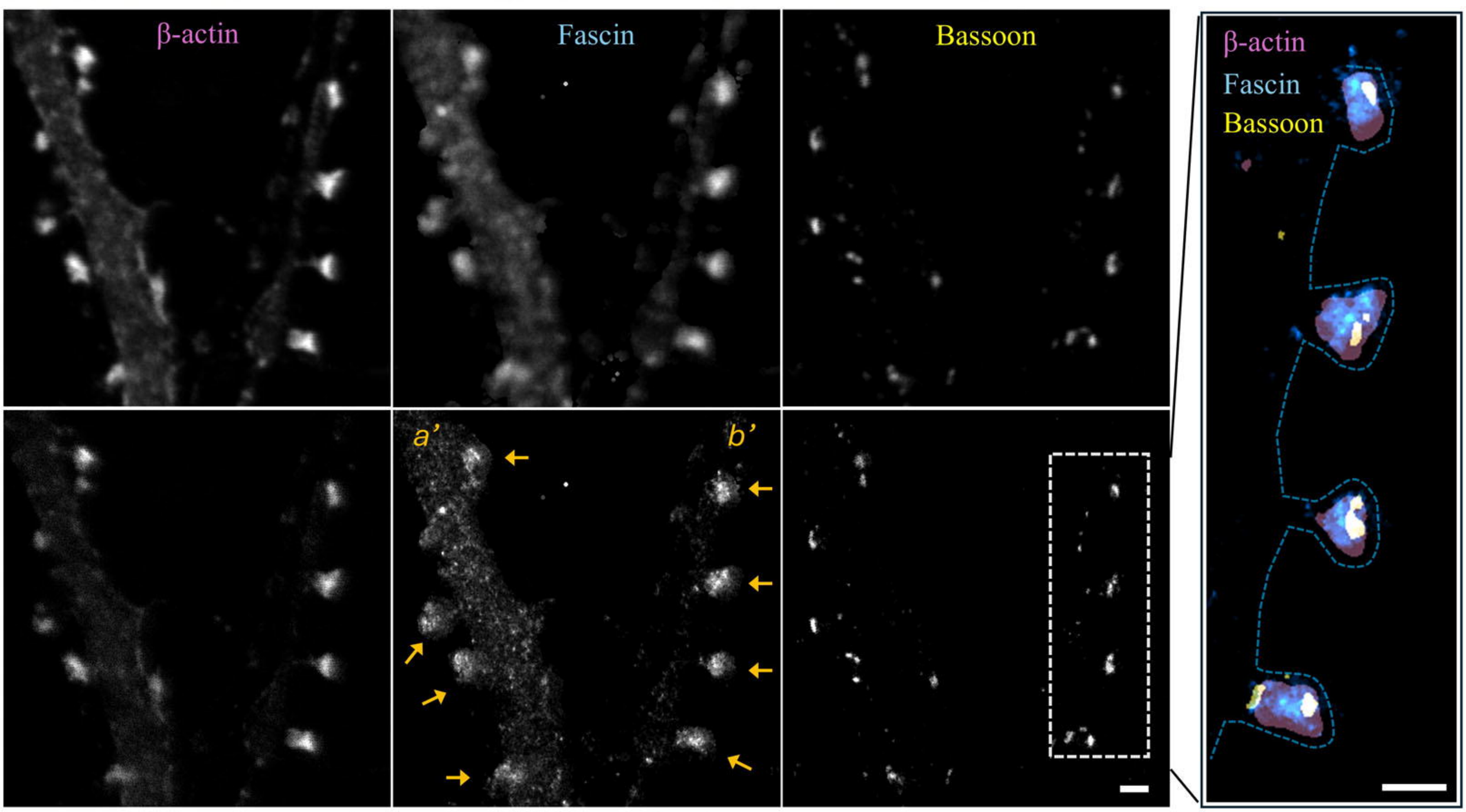
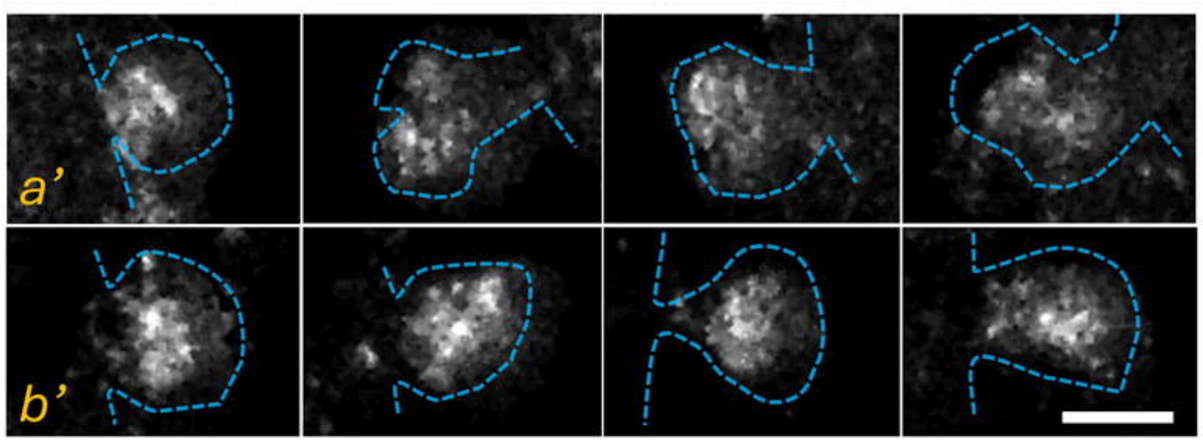
F-actin
a'
b'
Axon
GC


a'
b'

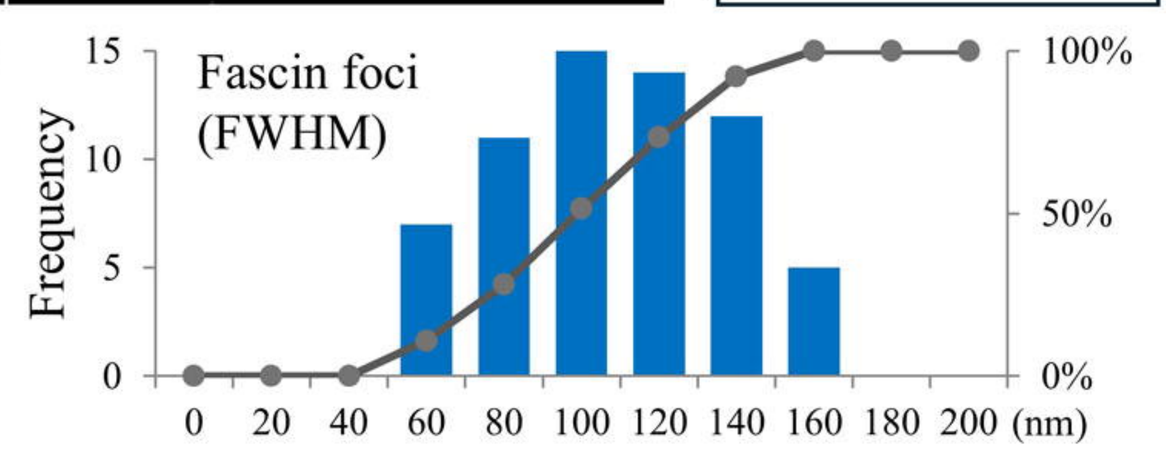
B

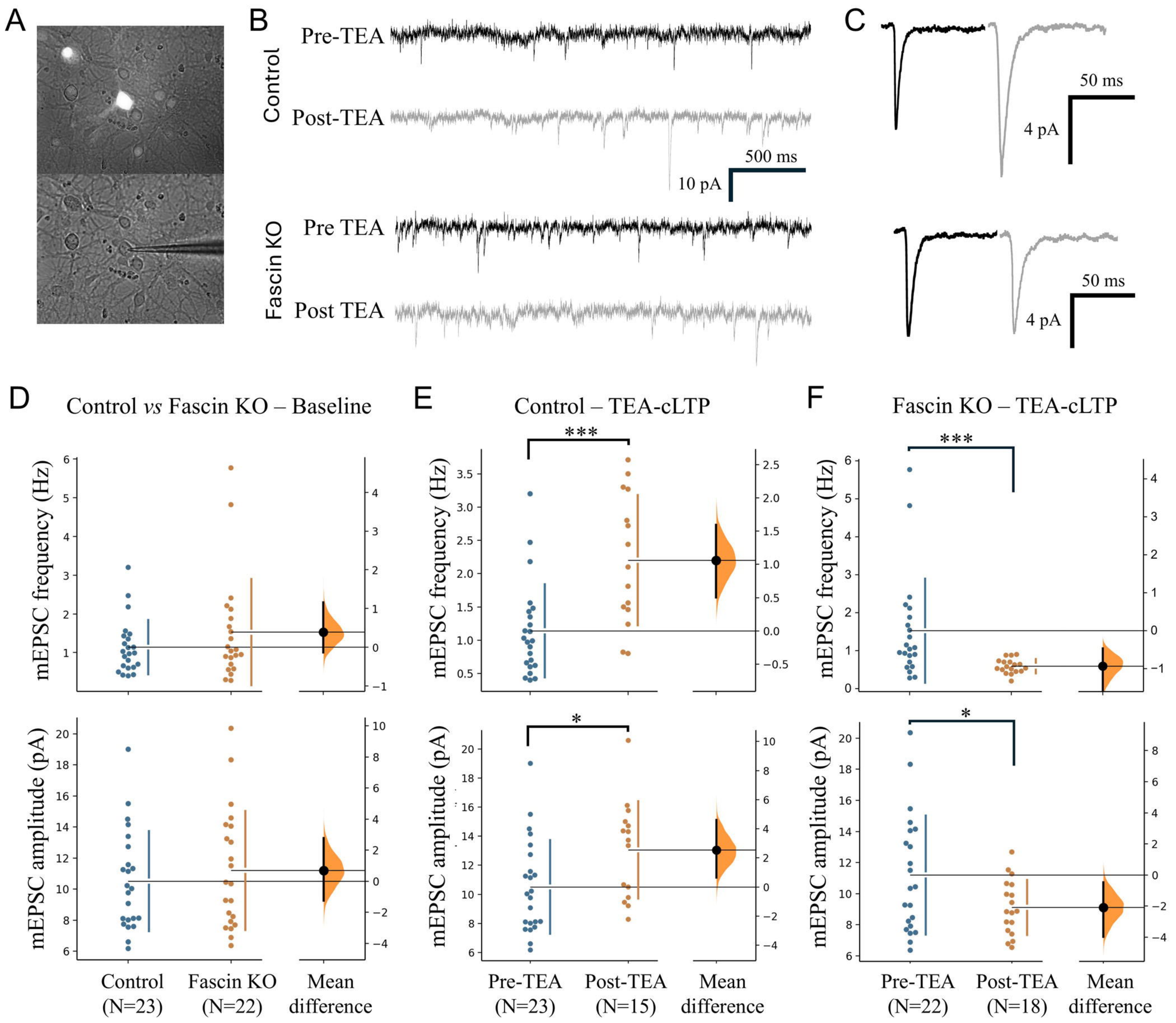

β-actin
Fascin
Bassoon
a'
b'
β-actin
Fascin
Bassoon

C


a'
b'

D


Fascin foci
(FWHM)
Frequency
15
10
5
0
100%
50%
0%
0
20
40
60
80
100
120
140
160
180
200
(nm)


A
B
Control
Pre-TEA
Post-TEA
500 ms
10 pA
Fascin KO
Pre TEA
Post TEA
C
50 ms
4 pA
50 ms
4 pA
D
Control vs Fascin KO – Baseline
mEPSC frequency (Hz)
mEPSC amplitude (pA)
Control (N=23)
Fascin KO (N=22)
Mean difference
E
Control – TEA-cLTP
***
*
mEPSC frequency (Hz)
mEPSC amplitude (pA)
Pre-TEA (N=23)
Post-TEA (N=15)
Mean difference
F
Fascin KO – TEA-cLTP
***
*
mEPSC frequency (Hz)
mEPSC amplitude (pA)
Pre-TEA (N=22)
Post-TEA (N=18)
Mean difference