Cytoskeleton

WILEY

CYTOSKELETON

PERSPECTIVE OPEN ACCESS

ARC Expands the DAAM1 Microexon-Mediated Actin–RHOA/ROCK Interplay

Patryk Poliński[1] | Marta Miret Cuesta[2,3] | Manuel Irimia[2,3,4]

[1]European Molecular Biology Laboratory, EMBL Barcelona, Barcelona, Spain | [2]Department of Medicine and Life Sciences (MELIS), Universitat Pompeu Fabra, Barcelona, Spain | [3]Centre for Genomic Regulation, Barcelona Institute of Science and Technology, Barcelona, Spain | [4]ICREA, Barcelona, Spain

Correspondence: Patryk Poliński (patrykpolinski@gmail.com) | Manuel Irimia (mirimia@gmail.com)

Received: 26 May 2025 | **Accepted:** 27 May 2025

Funding: The research has been funded by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (ERCCoG-LS2-101002275 to M.I.), Spanish Ministry of Science and Innovation (PID2020-115040GB-I00 to M.I.), the European Union's Horizon 2020 research and innovation program under grant agreements No. 721890 to P.P.

Keywords: alternative splicing | ARC | formin | synapse

ABSTRACT

Actin cytoskeleton and its dynamics play a crucial role in synaptic function, influencing dendritic spines' structural and functional plasticity. Recent findings unveiled the significance of alternative splicing of a neural-specific microexon in DAAM1 in modulating actin's role in synaptic processes. This article discusses the impact of this microexon on actin polymerization, the RHOA/ROCK signaling pathway, and cognitive functions. Furthermore, we present new results that reveal a more complex scenario involving the upregulation of the activity-regulated cytoskeleton-associated protein (ARC) protein in DAAM1 microexon KO models, which may further affect synaptic function and cognition.

1 | Introduction

Actin is a key cytoskeleton component, facilitating the structural changes necessary for cellular plasticity. This is particularly important in neurons, where it underlies the structural and functional plasticity of synapses. Actin filaments are essential for the formation and remodeling of dendritic spines, the sites responsible for receiving synaptic signals (Cingolani and Goda 2008). Recent research has underscored the importance of actin dynamics in synaptic function, with implications for cognitive processes such as learning and memory (Lamprecht 2021; McLeod and Salinas 2018). In these processes, the Rho family of small GTPases play crucial roles, as they regulate the number of dendritic spines by managing molecules needed for building the actin cytoskeleton (Zhang et al. 2021). One group of such molecules is formins, some of which interact directly with RHOA and are essential for actin nucleation and dynamics (Mattila and Lappalainen 2008; Pollard 2016). Formins are a diverse family characterized by a conserved formin-homology-2 (FH2) domain responsible for actin binding. Actin binding requires the release of the autoinhibitory interactions, which are controlled by the RHOA/ROCK signaling, and the subsequent dimerization of FH2 domains (Habas et al. 2001; Liu et al. 2008; Schönichen and Geyer 2010).

In our recent work (Poliński et al. 2025), we investigated how alternative splicing could regulate formin proteins in the brain. In particular, we focused on microexons, evolutionarily conserved and neuron-specific short coding sequences (3–30 nt long) that can be selectively included or excluded from mRNA transcripts (Irimia et al. 2014). Alternative splicing of microexons contributes to the diversification

Patryk Poliński and Manuel Irimia jointly supervised this work.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2025 The Author(s). *Cytoskeleton* published by Wiley Periodicals LLC.

and specialization of protein functions in the brain and was previously found to be enriched in actin-related processes (Irimia et al. 2014; Quesnel-Vallières et al. 2016). Moreover, microexons have been linked to cognitive dysfunction and autism spectrum disorder, further highlighting their importance (Irimia et al. 2014; Quesnel-Vallières et al. 2016; Parras et al. 2018; Gonatopoulos-Pournatzis et al. 2018, 2020).

We found several microexons in formin genes (*DAAM1*, *FMNL1*, and *FMNL2*), potentially modulating their protein functions in neurons. Among these, we focused on a strictly neuronal-specific, 9-amino acid microexon in DAAM1 (hereafter Daam1-MIC). The inclusion of this microexon extends the linker region of the FH2 domain, directly affecting DAAM1's capabilities for actin binding, polymerization, and fiber formation. Moreover, we find that the physiological impact of these quantitative and qualitative changes in actin cytoskeleton dynamics are further amplified by a decreased interaction of DAAM1 with RHOA upon the depletion of Daam1-MIC. Microexon-driven changes in DAAM1, RHOA, and the actin cytoskeleton interaction and dynamics lead to hyperactivation of the RHOA/ROCK signaling cascade, and cumulatively contribute to various phenotypes, including decreased number of learning spines in neurons, reduced long-term potentiation (LTP), and cognition defects (Poliński et al. 2025).

Extending our recent findings, we investigated the protein levels of immediate early genes (IEGs) associated with neuronal activation and plasticity. In particular, activity-regulated cytoskeleton-associated protein (ARC) is known to regulate the actin cytoskeleton and to control spine morphology during the LTP and long-term depression (LTD) processes (Zhang and Bramham 2021). Surprisingly, immunocytochemistry assays in in vitro differentiated glutamatergic neurons revealed consistently higher levels of ARC in Daam1-MIC KO compared to WT cells, whereas no changes for cFOS or EGR1 IEGs were observed (Figure 1a,b). In line with these results, we observed significantly higher ARC protein-positive nuclei in the hippocampal dentate gyrus of Daam1-MIC KO mice under basal conditions (Figure 1c,d). As the hippocampus is a brain unit responsible for cognitive processing, we investigated the IEG level changes upon learning. Interestingly, analysis of IEGs performed 1.5 h after the learning process (expression peak) showed clear downregulation of cFOS in KO mice, consistent with their decreased learning performance, but not of ARC protein (Figure 1i,j). These results point toward alterations not solely in actin polymerization and RHOA/ROCK signaling, but also misregulation of the essential IEGs, particularly the ARC protein (Figure 1f).

What leads to the increased ARC protein levels upon Daam1-MIC KO? The translation of ARC is regulated locally in dendritic spines (Newpher et al. 2018), and newly synthesized ARC impacts actin dynamics by interaction with many actin-binding proteins and other actin-regulating proteins (e.g., cofilin, CaMKIIβ, and drebrin A) (Messaoudi et al. 2007; Nakahata and Yasuda 2018; Okuno et al. 2012). Moreover, during Arc-dependent memory consolidation, local ARC protein translation depends on actin levels (Huang et al. 2007). Our data consistently showed increased ARC protein levels in basal conditions upon Daam1-MIC removal (Figure 1), with no significant changes at the mRNA level based on RNA-seq data (Poliński et al. 2025). Thus, it is plausible that enhanced actin bundling upon microexon removal creates the platform for more efficient *Arc* mRNA docking in dendritic spines and hence abnormally higher ARC translation in basal conditions (Figure 1f). Moreover, although the interplay between the RHO/ROCK signaling pathway and the ARC protein has not been explored yet, given that both play crucial roles in the formation and functioning of dendritic spines, they may affect each other.

Interestingly, newly synthesized ARC accumulates in nonstimulated spines and suppresses synaptic potentiation by decreasing the amounts of AMPA receptors in the postsynaptic surface (Okuno et al. 2012), in line with a role for ARC expression in inhibiting LTP (Plath et al. 2006; Verde et al. 2006). Thus, the increased expression of ARC may also contribute to the overall Daam1-MIC cellular and memory phenotypes. In this regard, RhoA also plays a role in regulating AMPA receptor trafficking, where activation of RhoA is associated with decreased surface AMPA receptors (Shen et al. 2020). Altogether, these data suggest numerous molecular interplays between RhoA, ARC, and the actin cytoskeleton that could determine the fate of dendritic spines and affect LTP.

In summary, our work revealed that *Daam1* is subject to tight posttranscriptional regulation by a highly neural-specific and evolutionarily conserved microexon that impacts actin polymerization via modulation of its FH2 domain. Moreover, two additional molecular phenotypes observed in Daam1-MIC KO neurons, increased RhoA activity and misregulation of basal ARC protein levels, provide additional insights into the underlying mechanisms of impaired memory formation. These results also show that the interplay between actin dynamics, alternative splicing, and synaptic function is a promising area of research that holds potential for advancing our understanding of learning and memory processes and their associated neurological disorders.

2 | Material and Methods

2.1 | Immunofluorescence Staining and Confocal Imaging of Cultured Neurons

Neuronal differentiation from mESCs was done following the protocol reported by Poliński et al. 2025, based on (Bibel et al. 2004, 2007). For immunofluorescence assays, cells on DIV21 were washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde in PBS for 10 min, permeabilized in 0.3% Triton X-100 in PBS for 10 min, blocked for 1 h in 0.3% Triton X-100, 3% bovine serum albumin (BSA) in PBS, and incubated in primary antibodies (cFos (guinea pig, 226,004, Synaptic Systems), Egr1 (rabbit, Sc-110, Santa Cruz), Arc (mouse, C-7 sc-17,839, Santa Cruz)) at 4°C overnight with shaking. Following this incubation, cells were incubated with the corresponding secondary antibodies (anti-Rabbit IgG—Alexa Fluor 488 (A-11034), anti-Guinea Pig IgG—Alexa Fluor 555(A-21435), anti-Mouse IgG—Alexa Fluor 647 (A-31571),

19493592, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cm.22051 by Universitat Pompeu Fabra, Wiley Online Library on [22/07/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

all Thermo Fisher Scientific) for 1 h at room temperature and mounted in FluoroShield with DAPI (Sigma, F6057-20 mL) for imaging. Images were taken on an SP8 confocal microscope (CRG, Advanced Light Microscopy Unit) using identical settings for each condition in each experiment. A dry 20× objective was used to image the whole span of the neuronal culture (17–23 μm, Z-step size 1 μm, zoom factor 2.5). Confocal sections were Z-stack projected with maximum intensity selection and analyzed in ImageJ/Fiji software. Immediate early genes positive nuclei were counted using the Analyze Particles plugin (nuclei size at least 25 pixels) and normalized to DAPI-positive nuclei.

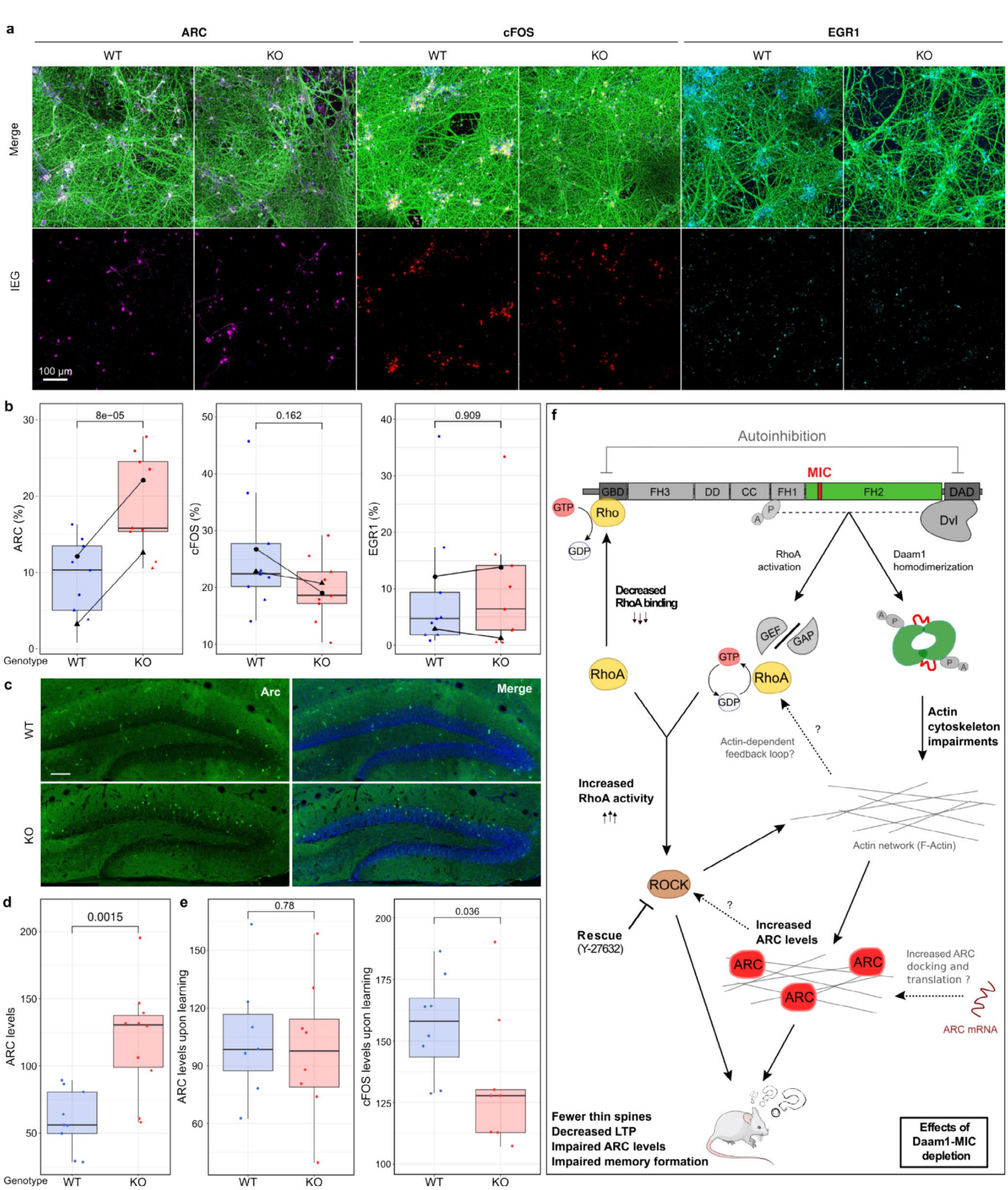

FIGURE 1 | Legend on next page.

19493592, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cm.22051 by Universitat Pompeu Fabra, Wiley Online Library on [22/07/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

FIGURE 1 | ARC protein expands the feedback loop between Actin, RHOA/ROCK signaling cascade, and the DAAM1 microexon. (a) Representative immunocytochemistry assay images of mature glutamatergic neurons (day in vitro 21; DIV21), stained with neuronal morphology marker β3-Tubulin (green) and IEG-positive nuclei for ARC (magenta), cFOS (red), and EGR1 (cyan). (b) Percentage quantification of ARC, cFOS, and EGR1-positive neuronal nuclei normalized to their total number based on DAPI (not shown). One dot represents one cell line average, for which 3–4 randomly selected regions of interest were analyzed. Different point shapes represent biological replicates. p values from a two-way ANOVA test with replicate and genotype as factors. (c) Representative images of ARC immunohistochemistry (green) and DAPI-stained nuclei (blue) in sections of the dentate gyrus of the hippocampus from PD21 mice. (d,e) Quantifications of IEG-positive nuclei in the hippocampal dentate gyrus across images normalized to the dentate gyrus size for ARC in basal conditions (d), or 1.5 h after learning (e) with ARC (left panel) and cFOS (right panel). p values from two-sided Wilcoxon rank-sum tests. One dot represents one animal for which 3–6 coronal views of the hippocampus were analyzed. (f) Overview of the phenotypes observed following the removal of Daam1-MIC and their connection to ARC and the RHOA/ROCK signaling pathway (Habas et al. 2001; Liu et al. 2008; Schönichen and Geyer 2010). DVL interacts with DAAM1, which removes its autoinhibition. This is further enhanced by additional interactions with RHOA. Active DAAM1 triggers RHOA activation via an unclear mechanism linked to DAAM1's C-terminal region (dashed line) and does not rely on direct binding between RHOA and DAAM1. Microexon removal reduces RHOA binding to DAAM1's N-terminal region. This is proposed to lead to reduced hydrolysis of RhoA-GTP, which increases the pool of active RHOA and, in turn, hyperactivates the RHOA/ROCK signaling cascade. Impaired actin cytoskeleton presumably enhances local translation of ARC mRNA and increases ARC protein levels. Arrows show the direction of the event. The dotted arrow suggests a potential, unknown feedback loop involving actin polymerization, ARC levels, and RHOA/ROCK pathway activation. ARC, Activity-regulated cytoskeleton-associated protein; GAP, GTPase-activating protein; GEF, Guanine nucleotide exchange factor; GTP, Guanosine triphosphate; GDP, Guanosine diphosphate.

2.2 | Immunohistochemical Imaging of the Mouse Hippocampus

Mouse hippocampal sections were prepared as previously described (Van Hoeymissen et al. 2020). In brief, mice were euthanized with CO2, perfused transcardially with 0.1 M PBS, followed by 4% paraformaldehyde in PBS until tissues were completely cleared of blood. Fixed brains were extracted and stored in 4% paraformaldehyde at 4°C for 24 h, and in sucrose 30% with 0.01% azide in PBS for the following 24 h. Prepared tissues were cut into 40-μm coronal sections, in serial order throughout the dorsal hippocampus (Bregma sections between −1.34 mm and −2.54 mm). Sectioning was performed by an in-house Tissue Engineering Unit (CRG). Immunohistochemistry was done by tissue permeabilization in 0.5% Triton X-100 in PBS for 15 min × 3 times, blocked for 2 h in 10% normal goat serum (NGS) in PBS albumin, and incubated with primary antibodies (cFos (guinea pig, 226,004, Synaptic Systems), Arc (mouse, C-7 sc-17,839, Santa Cruz)) in 5% NGS, Tween 0.5% in PBS at 4°C overnight. Following this incubation, cells were incubated with the corresponding secondary antibodies (anti-Guinea Pig IgG—Alexa Fluor 555 (A-21435), anti-Mouse IgG—Alexa Fluor 647 (A-31571), both Thermo Fisher Scientific) for 2 h at room temperature. Washing steps were repeated (for 15 min × 3 times), and sections were mounted in FluoroShield with DAPI for imaging. Images were taken on a confocal microscope (SP8 Leica; CRG, Advanced Light Microscopy Unit) using identical settings for each condition in a given experiment with a dry 20× objective. A single middle plane of each section was imaged, and the images were analyzed in ImageJ/Fiji software. DG size was marked manually with Polygon selections using DAPI-stained nuclei as a region of reference marker and consecutively quantified with the Measure plugin.

Acknowledgments

The research has been funded by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (ERCCoG-LS2-101002275 to M.I.), Spanish Ministry of Science and Innovation (PID2020-115040GB-I00 to M.I.), the European Union's Horizon 2020 research and innovation program under grant agreements No. 721890 to P.P.

Conflicts of Interest

The authors declare no conflicts of interest.

References

Bibel, M., J. Richter, E. Lacroix, and Y.-A. Barde. 2007. "Generation of a Defined and Uniform Population of CNS Progenitors and Neurons From Mouse Embryonic Stem Cells." *Nature Protocols* 2, no. 5: 1034–1043. https://doi.org/10.1038/nprot.2007.147.

Bibel, M., J. Richter, K. Schrenk, et al. 2004. "Differentiation of Mouse Embryonic Stem Cells Into a Defined Neuronal Lineage." *Nature Neuroscience* 7: 1003–1009. https://doi.org/10.1038/nn1301.

Cingolani, L. A., and Y. Goda. 2008. "Actin in Action: The Interplay Between the Actin Cytoskeleton and Synaptic Efficacy." *Nature Reviews Neuroscience* 9, no. 5: 344–356. https://doi.org/10.1038/nrn2373.

Gonatopoulos-Pournatzis, T., R. Niibori, E. W. Salter, et al. 2020. "Autism-Misregulated eIF4G Microexons Control Synaptic Translation and Higher Order Cognitive Functions." *Molecular Cell* 77, no. 6: 1176–1192.e16. https://doi.org/10.1016/j.molcel.2020.01.006.

Gonatopoulos-Pournatzis, T., M. Wu, U. Braunschweig, et al. 2018. "Genome-Wide CRISPR-Cas9 Interrogation of Splicing Networks Reveals a Mechanism for Recognition of Autism-Misregulated Neuronal Microexons." *Molecular Cell* 72, no. 3: 510–524.e12. https://doi.org/10.1016/j.molcel.2018.10.008.

Habas, R., Y. Kato, and X. He. 2001. "Wnt/Frizzled Activation of Rho Regulates Vertebrate Gastrulation and Requires a Novel Formin Homology Protein Daam1." *Cell* 107, no. 7: 843–854.

Huang, F., J. K. Chotiner, and O. Steward. 2007. "Actin Polymerization and ERK Phosphorylation Are Required for arc/Arg3.1 mRNA Targeting to Activated Synaptic Sites on Dendrites." *Journal of Neuroscience* 27, no. 34: 9054–9067. https://doi.org/10.1523/JNEUROSCI.2410-07.2007.

Irimia, M., R. J. Weatheritt, J. D. Ellis, et al. 2014. "A Highly Conserved Program of Neuronal Microexons Is Misregulated in Autistic Brains." *Cell* 159, no. 7: 1511–1523. https://doi.org/10.1016/j.cell.2014.11.035.

Lamprecht, R. 2021. "Actin Cytoskeleton Role in the Maintenance of Neuronal Morphology and Long-Term Memory." *Cells* 10, no. 7: 1795. https://doi.org/10.3390/cells10071795.

19493592, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cm.22051 by Universitat Pompeu Fabra, Wiley Online Library on [22/07/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Liu, W., A. Sato, D. Khadka, et al. 2008. "Mechanism of Activation of the Formin Protein Daam1." *Proceedings of the National Academy of Sciences of the United States of America* 105, no. 1: 210–215.

Mattila, P. K., and P. Lappalainen. 2008. "Filopodia: Molecular Architecture and Cellular Functions." *Nature Reviews Molecular Cell Biology* 9: 446–454. https://doi.org/10.1038/nrm2406.

McLeod, F., and P. C. Salinas. 2018. "Wnt Proteins as Modulators of Synaptic Plasticity." *Current Opinion in Neurobiology* 53: 90–95. https://doi.org/10.1016/j.conb.2018.06.003.

Messaoudi, E., T. Kanhema, J. Soule, et al. 2007. "Sustained arc/Arg3.1 Synthesis Controls Long-Term Potentiation Consolidation Through Regulation of Local Actin Polymerization in the Dentate Gyrus In Vivo." *Journal of Neuroscience* 27, no. 39: 10445–10455. https://doi.org/10.1523/JNEUROSCI.2883-07.2007.

Nakahata, Y., and R. Yasuda. 2018. "Plasticity of Spine Structure: Local Signaling, Translation and Cytoskeletal Reorganization." *Frontiers in Synaptic Neuroscience* 10: 29. https://doi.org/10.3389/fnsyn.2018.00029.

Newpher, T. M., S. Harris, J. Pringle, C. Hamilton, and S. Soderling. 2018. "Regulation of Spine Structural Plasticity by arc/Arg3.1." *Seminars in Cell & Developmental Biology* 77: 25–32. https://doi.org/10.1016/j.semcdb.2017.09.022.

Okuno, H., K. Akashi, Y. Ishii, et al. 2012. "Inverse Synaptic Tagging of Inactive Synapses via Dynamic Interaction of Arc/Arg3.1 With CaMKIIβ." *Cell* 149, no. 4: 886–898. https://doi.org/10.1016/j.cell.2012.02.062.

Parras, A., H. Anta, M. Santos-Galindo, et al. 2018. "Autism-Like Phenotype and Risk Gene mRNA Deadenylation by CPEB4 Mis-Splicing." *Nature* 560, no. 7719: 441–446. https://doi.org/10.1038/s41586-018-0423-5.

Plath, N., O. Ohana, B. Dammermann, et al. 2006. "Arc/Arg3.1 Is Essential for the Consolidation of Synaptic Plasticity and Memories." *Neuron* 52, no. 3: 437–444. https://doi.org/10.1016/j.neuron.2006.08.024.

Poliński, P., M. Miret Cuesta, A. Zamora-Moratalla, et al. 2025. "A Highly Conserved Neuronal Microexon in DAAM1 Controls Actin Dynamics, RHOA/ROCK Signaling, and Memory Formation." *Nature Communications* 16: 4210. https://doi.org/10.1038/s41467-025-59430-w.

Pollard, T. D. 2016. "Actin and Actin-Binding Proteins." *Cold Spring Harbor Perspectives in Biology* 8, no. 8: a018226. https://doi.org/10.1101/cshperspect.a018226.

Quesnel-Vallières, M., Z. Dargaei, M. Irimia, et al. 2016. "Misregulation of an Activity-Dependent Splicing Network as a Common Mechanism Underlying Autism Spectrum Disorders." *Molecular Cell* 64, no. 6: 1023–1034. https://doi.org/10.1016/j.molcel.2016.11.033.

Schönichen, A., and M. Geyer. 2010. "Fifteen Formins for an Actin Filament: A Molecular View on the Regulation of Human Formins." *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1803, no. 2: 152–163. https://doi.org/10.1016/j.bbamcr.2010.01.014.

Shen, W., M. B. C. Kilander, M. S. Bridi, et al. 2020. "Tomosyn Regulates the Small RhoA GTPase to Control the Dendritic Stability of Neurons and the Surface Expression of AMPA Receptors." *Journal of Neuroscience Research* 98, no. 6: 1213–1231. https://doi.org/10.1002/jnr.24608.

Van Hoeymissen, E., K. Philippaert, R. Vennekens, J. Vriens, and K. Held. 2020. "Horizontal Hippocampal Slices of the Mouse Brain." *JoVE (Journal of Visualized Experiments)* 22: 163. https://doi.org/10.3791/61753.

Verde, R., M. Emiliano, J. Lee-Osbourne, P. F. Worley, R. Malinow, and H. T. Cline. 2006. "Increased Expression of the Immediate-Early Gene Arc/Arg3.1 Reduces AMPA Receptor-Mediated Synaptic Transmission." *Neuron* 52, no. 3: 461–474. https://doi.org/10.1016/j.neuron.2006.09.031.

Zhang, H., Y. Ben Zablah, H. Zhang, and Z. Jia. 2021. "Rho Signaling in Synaptic Plasticity, Memory, and Brain Disorders." *Frontiers in Cell and Developmental Biology* 9: 729076. https://doi.org/10.3389/fcell.2021.729076.

Zhang, H., and C. R. Bramham. 2021. "Arc/Arg3.1 Function in Long-Term Synaptic Plasticity: Emerging Mechanisms and Unresolved Issues." *European Journal of Neuroscience* 54, no. 8: 6696–6712. https://doi.org/10.1111/ejn.14958.

19493592, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/cm.22051 by Universitat Pompeu Fabra, Wiley Online Library on [22/07/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License