A recurrent, *de novo* pathogenic variant in *ARPC4* disrupts actin filament formation and causes microcephaly and speech delay

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Summary

We report seven affected individuals from six families with a recurrent, *de novo* variant in the *ARPC4* gene (c.472C>T [p.Arg158Cys (GenBank: NM_005718.4)]). Core features in affected individuals include microcephaly, mild motor delays, and significant speech impairment. ARPC4 is a core subunit of the actin-related protein (ARP2/3) complex, which catalyzes the formation of F-actin networks. We show that the recurrent ARPC4 missense change is associated with a decreased amount of F-actin in cells from two affected individuals. Taken together, our results implicate heterozygous *ARPC4* missense variants as a cause of neurodevelopmental disorders and microcephaly.

The actin cytoskeleton plays an important role in many developmental processes in the brain, including cell division and proliferation, cell and axon migration, and synaptic plasticity. Disruption of actin dynamics (polymerization and depolymerization) can lead to abnormal brain development, including periventricular heterotopia[1] and microcephaly.[2] The actin cytoskeleton is composed of highly branched networks of actin filaments (F-actin), which are assembled from free actin monomers (G-actin). The nucleation of new F-actin filaments is the rate-limiting step of F-actin polymerization.[3] The actin-related protein-2/3 (ARP2/3) complex is a highly conserved actin-nucleating complex that binds to the side of existing actin filaments and nucleates the formation of new daughter filaments to form branched actin networks.[4] As the only nucleator of branched actin filaments, it has a central role in both F-actin polymerization and actin cytoskeleton organization.

The ARP2/3 complex consists of seven subunits: two actin-related proteins (ARP2 and ARP3) and five ARP2/3 complex polypeptides (ARPC1B and ARPC2-5). ARPC4 is essential for the structural integrity of the ARP2/3 complex function. It binds to ARPC2, forming the structural core of the complex around which the other subunits bind.[5] Consistent with its central role in actin cytoskeleton formation, knockout of *Arpc4* in mice is embryonic lethal.

Conditional knockout of *Arpc4* in the epidermis of mice leads to a reduction of F-actin levels and morphological changes in keratinocytes, including the absence of lamellipodia and ruffles, congruent with the disruption of the actin cytoskeleton.[6]

Through clinical exome sequencing, we identified a *de novo* variant of uncertain significance in the *ARPC4* gene (hg19: chr3: g.9845668C>T [c.472C>T; p.Arg158Cys (GenBank: NM_005718.4)]) in a young girl (individual 1a) with primary microcephaly, mild motor delays, and significant speech delay. Her younger brother (individual 1b) presented similarly, with mild microcephaly and motor delays; targeted testing confirmed that he had the same *de novo* variant, suggesting gonadal mosaicism in a parent. Using targeted deep sequencing,[7] we confirmed low-level mosaicism (0.7% variant allele frequency) in their mother. Using GeneMatcher,[8] we identified five additional individuals with the same *de novo* variant. The variant is absent from control populations (gnomADv2.1.1). *In silico* analysis, which includes protein predictors and evolutionary conservation, supports a deleterious effect. The Institutional Review Board of the University of Washington approved this study. Parents of affected children included in this study provided informed consent.

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All seven affected individuals with the recurrent variant presented with motor delays noted by 1 year (Table 1). Of the seven individuals who are at least 2 years old, five can walk independently. Speech is significantly impacted in all affected individuals, with most using only short (three-word) phrases even at 7–11 years old; two individuals successfully use assistive communication devices. Three of four individuals who are school-aged require special education settings and exhibit hyperactivity. Eye abnormalities were common and included strabismus (5/7), central visual impairment (2/7), and congenital cataracts (1/7). Nearly all (6/7) individuals have microcephaly, with head size ranging from 2.1 to 5 standard deviations below the mean. One individual (individual 5) exhibited slowed head growth, with an occipital frontal circumference (OFC) that dropped from 42nd percentile to 15th percentile by age 10 years. Facial features are shown in Figure 1.

The ARPC2 and ARPC4 proteins form the primary site at which the ARP2/3 complex binds to the side of an existing F-actin filament. This interface is critical for the nucleation of new daughter F-actin filaments and the stability of the resulting F-actin branch network. The R158 residue in ARPC4 is one of several amino acids located at the interface between the ARP2/3 complex and F-actin and is predicted to interact with the surface of F-actin through the formation of a salt bridge.[9] The p.Arg158Cys variant replaces a positively charged arginine residue with an uncharged cystine residue, preventing salt bridge formation. As such, the p.Arg158Cys recurrent variant is predicted to have an adverse effect on the ARP2/3 complex's ability to bind F-actin.

Though there are no recognizable functional domains in ARPC4, and it is overall tolerant to missense variants (gnomADv2.1.1, $Z = 2.21$), the recurrence of the p. Arg158Cys variant in seven individuals with similar phenotypes strongly supported its implication in human disease. To verify that the recurrent ARPC4:p.Arg158Cys variant impacts protein function, we evaluated F-actin abundance in fibroblasts from affected and unaffected individuals. Primary fibroblast cultures were established from skin biopsies from the two affected siblings with the recurrent p.Arg158Cys variant (1a and 1b), as well as from skin biopsies from two unrelated, unaffected control subjects. Fibroblasts were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. All experiments were performed on cells whose passage number did not exceed 10. Immunostaining for F-actin was performed on 2% paraformaldehyde-fixed fibroblasts according to standard protocols using actin stain conjugated to Alexa-555 (100 nM, Cytoskeleton) incubated for 30 min at room temperature. DAPI (4′,6-diamidino-2-phenylindole) was used to visualize the nuclei. Fluorescent images were captured using a Zeiss LSM 880 laser scanning microscope equipped with Airyscan technology. All immunostaining was performed in triplicate. F-actin abundance was measured as the fluorescence intensity per cell normalized to the cell area (n = 155–193 per individual). We observed a 43% decrease in F-actin abundance in the cells from affected individuals compared to control individuals (Figure 2), confirming that the p.Arg158Cys variant disrupts ARPC4 function and F-actin network formation. These results, along with the fact that there are no loss-of-function variants in *ARPC4* in the gnomAD database, suggest that the p.Arg158Cys variant causes a loss of ARPC4 function. We cannot rule out a dominant negative effect, though this may be less likely, as complete lack of ARPC4 is embryonic lethal in mice.

Primary microcephaly, which was observed in 6/7 individuals in this study, is caused by a disruption in the proliferation of the neuronal progenitor cells, which leads to the depletion of the neural progenitor pool, an overall reduction in neuron production, and ultimately a smaller-than-expected brain. Many of the genes associated with primary microcephaly encode centrosome proteins, although other pathways, such as DNA repair, have also been implicated.[2] The actin cytoskeleton is crucial to neuronal progenitor cell proliferation. It is a major component of the cleavage furrow, and chemical inhibition of actin filaments leads to cytokinesis failure and cell cycle arrest.[10] Additionally, neuronal progenitor cell differentiation begins through asymmetrical cell division, where the actin cytoskeleton coordinates the enrichment of cell fate determining proteins into one daughter cell, which escapes proliferation and enters neurogenesis.[11] Disruption of this actin-dependent process can lead to premature differentiation, depleting the pool of neuronal progenitor cells.

Apart from its role in cell division, the actin cytoskeleton is crucial for many other developmental processes in the brain. Remodeling of the actin cytoskeleton provides the mechanical force necessary for cell migration and modulation of cell shape, processes that play a critical role in neuron migration, axon guidance, and the regulation of synapse morphology.[12] The importance of the ARP2/3 complex is most evident in the dendritic spines of excitatory neurons, where it is responsible for the production of a dense network of branched actin. Loss of the Arp2/3 complex from postnatal excitatory neurons led to the loss of plasticity of dendritic spines, a reduction in the number of spines, and behavioral abnormalities in mice.[13] At the excitatory synapses, primarily located in the dendritic spines, the ARP2/3-derived actin cytoskeleton facilitates signaling between the postsynaptic density scaffold proteins and various cell membrane and intracellular molecules, which is crucial for synaptic plasticity.[12] Through its central role maintaining and remodeling the actin cytoskeleton, the ARP2/3 complex influences neurodevelopment, cognitive performance, and social behavior.

Several other neurological disorders are due to pathogenic variants in genes involved in the formation and regulation of the actin cytoskeleton. *FLNA* (MIM: 300017), the leading cause of X-linked periventricular heterotopia, forms crosslinks between actin filaments to

Table 1. Summary of clinical features of individuals with *de novo* (p.Arg158Cys) variant in *ARPC4*

Individual (gender, current age)	Variant (protein)	Inheritance	OFC (age)	Motor development	Speech	Other
1a, (F, 9 years)	p.Arg158Cys	*de novo*[a]	−3.2 SD (7 months) −5 SD (9 years 4 months)	crawled at 10 months; walked at 18 months	expressive delays; 10 words at 2.5 years; at 4 years speech noted to be at 15-to 18-month level; 3-word phrases at 9 years	strabismus, accommodative esotropia; thumb-in-palm (splinted); ADHD, tantrums; facial telangiectasias
1b, (M, 7 years)	p.Arg158Cys	*de novo*[a]	−2.6 SD (7 months) −3 SD (7 years 5 months)	sat at 1 year; walked at 22 months	first words 18–24 months; 3-word phrases at 7 years; uses assistive communication device	ADHD; clasped thumbs, 5th finger camptodactyly (splinted); facial telangiectasias
2, (F, 6 years)	p.Arg158Cys	*de novo*	−2.8 SD (6 years 9 months)	sat at 12 months; walked at 14–16 months; pincer at 4 years	short phrases since 3 years	strabismus (surgery ×2)
3, (F, 14 months)	p.Arg158Cys	*de novo*	5% (14 months) −2.5 SD (26 months)	walked by 22 months; descends stairs at 26 months; turns pages; holds sippy cup	no expressive language at 26 months; uses assistive communication device	congenital cataracts, strabismus, CVI; thumb abduction; low RBC plasmalogens
4, (M, 2 years)	p.Arg158Cys	*de novo*	4% (birth) −3.8 SD (14 months) −2.7 SD (32 months)	sits at 20 months, attempting to crawl; palmar grasp (20 months); holds toys	babbling at 24 months; nonverbal at 35 months	CVI, myopic astigmatism; apraxia; hydronephrosis; short stature
5, (F, 11 years)	p.Arg158Cys	*de novo*	42% (birth) 15% (10 years 8 months)	sat at 9 months; crawled at 13 months; walked at 17 months	words by 2 years; uses phrases; articulation difficulties	intermittent exotropia; severe hyperactivity, impulsivity, aggression; scoliosis
6, (F, 4 years)	p.Arg158Cys	*de novo*	4% (birth) −2.1 SD (16 months)	moderate delays; not walking at 4 years	nonverbal at 4 years	strabismus; myelomeningocele, tethered cord; malplaced thumbs; pulmonic stenosis; L inguinal hernia; stereotypies; IQ 52

OFC, occipital frontal circumference; ADHD, attention deficit hyperactivity disorder; CVI, cortical visual impairment; F, female; M, male; RBC, red blood cell; SD, standard deviation.
[a]Maternal mosaicism (0.7%).

Figure 1. Facial photographs of affected individuals
(A) Individual 1a at 9 years 4 months.
(B and C) Individual 1b at 7 years 5 months.
(D and E) Individual 4 in infancy (D) and at 1 year (E).
(F) Individual 5.
(G–I) Individual 6. Shared features include thin upper lip, long palpebral fissures that are slightly upslanting, and mild micrognathia in some affected individuals.
(J) Magnetic resonance imaging (MRI) for individual 4 showing nonspecific prominence of ventricles, sulci, and subarachnoid spaces diffusely and incomplete inversion of hippocampi.

stabilize the actin cytoskeleton. *CAPZA2* (MIM: 601571), associated with intellectual disability and developmental delay, is a member of an F-actin capping complex and is necessary for the stabilization of F-actin filaments.[14] Biallelic, truncating variants in the F-actin nucleation factor *FMN2* (MIM: 606373) cause autosomal-recessive intellectual disability.[15] Disruption of *SHANK3* (MIM: 606230), which interacts with the ARP2/3 complex to stabilize F-actin, causes Phelan-McDermid syndrome, characterized by hypotonia, global developmental delay, autistic behaviors, and profound language delays.[16] *CYFIP2* (MIM: 606323), which is associated with developmental and epileptic encephalopathy, is a subunit of the WAVE-regulatory complex and promotes F-actin nucleation through activation of the ARP2/3 complex.[17] Even actin is implicated in neurological disease. Gain-of-function variants in *ACTB* (MIM: 102630) or *ACTG1* (MIM: 102560), which encode nearly identical actin subunits, cause a syndromic neurodevelopmental disorder characterized by developmental delay, intellectual disability, and internal organ abnormalities.[18] Since most actin regulatory proteins are broadly expressed,[19] the enrichment of neurological abnormalities in these disorders provides further evidence that the cells in the nervous system are particularly susceptible to disruption of the actin cytoskeleton.

In summary, we report seven individuals with the same recurrent, *de novo* variant in *ARPC4* and a neurodevelopmental disorder characterized by microcephaly, mild motor delays, and significant speech impairment. Identification of additional affected individuals will provide further insight into the range of neurological features associated with *ARPC4* and elucidate the full mutational spectrum in *ARPC4* capable of causing disease.

Data availability

This study collated results of clinical exome sequence and did not generate/analyze sequence data.

Consortia

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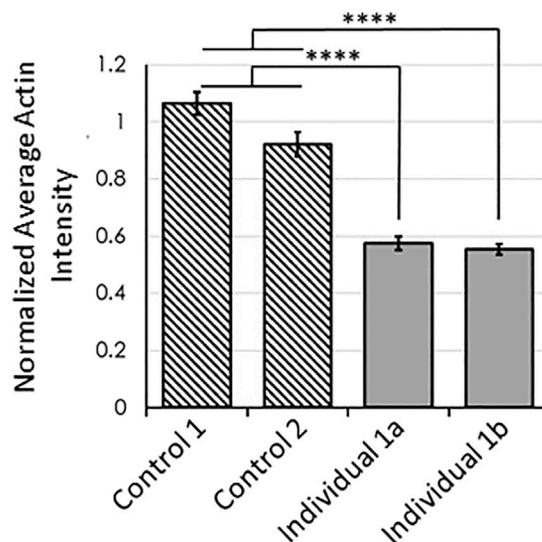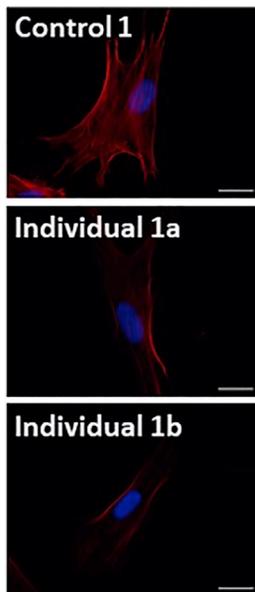
Figure 2. Reduced F-actin fibers in fibroblasts from affected individuals

Representative immunofluorescent images showing a reduction in the intensity of actin staining in the fibroblasts of affected individuals (1a and 1b) compared to unaffected control individuals (Ctrl). F-actin was quantified by measuring the average cellular actin fluorescent intensity. Error bars represent SEM. The scale bar represents 20 μm. The experiment was performed in triplicate with a combined n = 155–193 per individual; representative images shown. ****$p <$ 0.0001 by two-tailed unpaired Student's t test.

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Acknowledgments

Declaration of interests

K.G.M., T.S.S., and I.M.W. are employees of GeneDx, Inc. All other authors declare no competing interests.

Web resources

OMIM, https://www.omim.org

References

1. Lian, G., and Sheen, V.L. (2015). Cytoskeletal proteins in cortical development and disease: actin associated proteins in periventricular heterotopia. Front. Cell. Neurosci. *9*, 99.

2. Jayaraman, D., Bae, B.I., and Walsh, C.A. (2018). The Genetics of Primary Microcephaly. Annu. Rev. Genomics Hum. Genet. *19*, 177–200.

3. Pollard, T.D., and Borisy, G.G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. Cell *112*, 453–465.

4. Goley, E.D., and Welch, M.D. (2006). The ARP2/3 complex: an actin nucleator comes of age. Nat. Rev. Mol. Cell Biol. *7*, 713–726.

5. Gournier, H., Goley, E.D., Niederstrasser, H., Trinh, T., and Welch, M.D. (2001). Reconstitution of human Arp2/3 complex reveals critical roles of individual subunits in complex structure and activity. Mol. Cell *8*, 1041–1052.

6. van der Kammen, R., Song, J.Y., de Rink, I., Janssen, H., Madonna, S., Scarponi, C., Albanesi, C., Brugman, W., and Innocenti, M. (2017). Knockout of the Arp2/3 complex in epidermis causes a psoriasis-like disease hallmarked by hyper-activation of transcription factor Nrf2. Development *144*, 4588–4603.

7. Myers, C.T., Hollingsworth, G., Muir, A.M., Schneider, A.L., Thuesmunn, Z., Knupp, A., King, C., Lacroix, A., Mehaffey, M.G., Berkovic, S.F., et al. (2018). Parental Mosaicism in "De Novo" Epileptic Encephalopathies. N. Engl. J. Med. *378*, 1646–1648.

8. Sobreira, N., Schiettecatte, F., Valle, D., and Hamosh, A. (2015). GeneMatcher: a matching tool for connecting investigators with an interest in the same gene. Hum. Mutat. *36*, 928–930.

9. Goley, E.D., Rammohan, A., Znameroski, E.A., Firat-Karalar, E.N., Sept, D., and Welch, M.D. (2010). An actin-filament-binding interface on the Arp2/3 complex is critical for nucleation and branch stability. Proc. Natl. Acad. Sci. USA *107*, 8159–8164.

10. Lee, K., and Song, K. (2007). Actin dysfunction activates ERK1/2 and delays entry into mitosis in mammalian cells. Cell Cycle *6*, 1487–1495.

11. Knoblich, J.A. (2008). Mechanisms of asymmetric stem cell division. Cell *132*, 583–597.

12. Spence, E.F., and Soderling, S.H. (2015). Actin Out: Regulation of the Synaptic Cytoskeleton. J. Biol. Chem. *290*, 28613–28622.

13. Kim, I.H., Racz, B., Wang, H., Burianek, L., Weinberg, R., Yasuda, R., Wetsel, W.C., and Soderling, S.H. (2013). Disruption of Arp2/3 results in asymmetric structural plasticity of dendritic spines and progressive synaptic and behavioral abnormalities. J. Neurosci. *33*, 6081–6092.

14. Huang, Y., Mao, X., van Jaarsveld, R.H., Shu, L., Terhal, P.A., Jia, Z., Xi, H., Peng, Y., Yan, H., Yuan, S., et al. (2020). Variants in CAPZA2, a member of an F-actin capping complex, cause intellectual disability and developmental delay. Hum. Mol. Genet. *29*, 1537–1546.

15. Law, R., Dixon-Salazar, T., Jerber, J., Cai, N., Abbasi, A.A., Zaki, M.S., Mittal, K., Gabriel, S.B., Rafiq, M.A., Khan, V., et al. (2014). Biallelic truncating mutations in FMN2, encoding the actin-regulatory protein Formin 2, cause nonsyndromic autosomal-recessive intellectual disability. Am. J. Hum. Genet. *95*, 721–728.

16. Durand, C.M., Betancur, C., Boeckers, T.M., Bockmann, J., Chaste, P., Fauchereau, F., Nygren, G., Rastam, M., Gillberg, I.C., Anckarsäter, H., et al. (2007). Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. Nat. Genet. *39*, 25–27.

17. Nakashima, M., Kato, M., Aoto, K., Shiina, M., Belal, H., Mukaida, S., Kumada, S., Sato, A., Zerem, A., Lerman-Sagie, T., et al. (2018). De novo hotspot variants in CYFIP2 cause early-onset epileptic encephalopathy. Ann. Neurol. *83*, 794–806.

18. Cuvertino, S., Stuart, H.M., Chandler, K.E., Roberts, N.A., Armstrong, R., Bernardini, L., Bhaskar, S., Callewaert, B., Clayton-Smith, J., Davalillo, C.H., et al.; DDD Study (2017). ACTB Loss-of-Function Mutations Result in a Pleiotropic Developmental Disorder. Am. J. Hum. Genet. *101*, 1021–1033.

19. Consortium, G.T.; and GTEx Consortium (2013). The Genotype-Tissue Expression (GTEx) project. Nat. Genet. *45*, 580–585.