De novo mutations in the actin genes ACTB and ACTG1 cause Baraitser-Winter syndrome

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Brain malformations are individually rare but collectively common causes of developmental disabilities1–3. Many forms of malformation occur sporadically and are associated with reduced reproductive fitness, pointing to a causative role for de novo mutations4,5. Here, we report a study of Baraitser-Winter syndrome, a well-defined disorder characterized by distinct craniofacial features, ocular colobomata and neuronal migration defect6,7. Using whole-exome sequencing of three proband-parent trios, we identified de novo missense changes in the cytoplasmic actin-encoding genes ACTB and ACTG1 in one and two probands, respectively. Sequencing of both genes in 13 additional affected individuals identified disease-causing mutations in all probands, including two recurrent de novo alterations (ACTB, enconding p.Arg196His, and ACTG1, encoding p.Ser155Phe). Our results confirm that trio-based exome sequencing is a powerful approach to discover genes causing sporadic developmental disorders, emphasize the overlapping roles of cytoplasmic actin proteins in development and suggest that Baraitser-Winter syndrome is the predominant phenotype associated with mutation of these two genes.

Whole-exome sequencing has recently been used to detect disease-causing mutations underlying rare, sporadic syndromes that occur in only a few unrelated individuals8–10. However, this approach becomes more difficult in the presence of any locus heterogeneity. For disorders hypothesized to result from de novo mutations, a potentially more powerful alternative approach involves exome sequencing in parent-child trios, which should rapidly narrow the number of candidate mutations to a handful, in view of the rarity of de novo events in protein-coding sequences11,12. This strategy has been successfully applied to identify de novo point mutations in intellectual disability4, autism13 and schizophrenia14,15.

Baraitser-Winter syndrome is a rare but well-defined developmental disorder recognized by the combination of congenital ptosis, high-arched eyebrows, hypertelorism, ocular colobomata and a brain malformation consisting of anterior predominant lissencephaly. Other typical features include postnatal short stature and microcephaly, intellectual disability, seizures and hearing loss6,7,16–18. Neither familial recurrence nor consanguinity has been observed in any families, including the 18 reported here (Supplementary Note), and no pathogenic copy-number variants (CNVs) have been detected using chromosome microarrays. We therefore hypothesized that the genetic basis of Baraitser-Winter syndrome was likely to result from de novo point mutations, and we performed whole-exome sequencing in three probands (subject LP92-083 is shown in Fig. 1) and their unaffected parents using two different platforms. Exome capture and sequencing were performed using NimbleGen solution-based capture and Illumina sequencing for trio 1 (LP92-083) and the Agilent SureSelect target enrichment system with ABI SOLiD sequencing for trios 2 (58248) and 3 (58431) (Online Methods). Both platforms generated

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coverage of at least ten reads for more than 85% of the targeted exome, and we identified 22,591–29,685 genetic variants per proband (Table 1). As previously described\(^4\),\(^5\), we filtered variants to systematically identify candidate de novo events in each proband. Given the severe phenotype, we focused on protein-altering and splice-site variants absent from other exome data sets available locally or from the dbSNP or 1000 Genomes Project databases\(^9\). Similar to previous studies\(^4\),\(^13\),\(^15\), we identified two to six candidate de novo mutations per proband (Table 1 and Supplementary Table 1), all of which were tested by Sanger sequencing.

The two sequencing and analysis platforms converged to identify de novo missense changes in the ACTG1 gene encoding cytoplasmic γ-actin (NM_001614) in two probands and in ACTB encoding β-actin (NM_001101) in the third proband (Supplementary Fig. 1). We used Sanger sequencing to screen the coding sequence of both genes in 15 additional affected individuals and detected pathogenic mutations in one of these genes in all subjects. Altogether, we found ten mutations in ACTB and eight mutations in ACTG1 (Table 2). These mutations proved to be de novo in all 11 subjects with parental DNA available, and 6 of the 7 mutations identified in individuals for whom parental DNA was not available were identical to mutations shown to be de novo in the first group. Of note, eight individuals (44%) carried a mutation disrupting Arg196 of β-actin, with seven having the same nucleotide change (c.587G>A; p.Arg196His). These recurrent transitions occurred at a CpG dinucleotide, a site that is susceptible to deamination of methylcytosines\(^20\), thus suggesting a possible mechanism for the high frequency of this mutation. Another three individuals (17%) had a mutation disrupting Ser155 of cytoplasmic γ-actin.

We analyzed several control data sets to validate our findings and assess the number of coding variants in ACTB and ACTG1. These included whole-exome sequence data from 244 unrelated, healthy individuals of European descent (Online Methods and Supplementary Table 2) and genome-wide sequence data from 629 individuals available from the 1000 Genomes Pilot Project\(^19\) and ~2,500 individuals from the National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project (ESP) (Supplementary Tables 3 and 4). None of the mutations identified in individuals with Baraitser-Winter syndrome was present in this large data set. Furthermore, no CNVs encompassing ACTB or ACTG1 exons were observed in 2,349 control individuals (Online Methods). Notably, the ESP data set showed a ratio of synonymous to nonsynonymous substitutions of 79:1 for the covered portions of ACTB and ACTG1 combined. On a genome-wide scale\(^13\), this ratio is estimated to be ~1:1, thus indicating strong selection against nonsynonymous mutations in both genes. Unexpectedly, one of the ACTG1 de novo alterations (encoding p.Ala135Val) was recorded in dbSNP (rs11549190; Table 2). This variant was originally identified but not validated in large-scale sequencing of ESTs\(^21\). Given its absence in the aforementioned data sets, we conclude that this report was likely an error in dbSNP.

The actin family is composed of essential cytoskeletal proteins implicated in nearly all cellular processes\(^22\),\(^25\). Of the six human genes encoding actin, only ACTB and ACTG1 are ubiquitously expressed, with the remaining four being expressed primarily in muscle. The cytoplasmic β- and γ-actin proteins are highly conserved throughout evolution and are nearly identical to each other, differing by only four amino acids\(^26\) (Supplementary Fig. 2). Despite their extensive homology and expression in all cell types\(^27\),\(^2\), β- and γ-actin have been proposed to have physiological functions that are at least partially distinct, based on their relative spatial and temporal enrichment in neurons\(^28\) and other cell types\(^29,30\). In addition, homozygous deletion of these genes in mice has been described to result in different phenotypes: Actb\(^0\)/– mice are embryonic lethal, whereas Actg1\(^0\)/– mice have reduced viability, with some animals surviving to adulthood\(^25,31,32\). Previously reported heterozygous ACTB and ACTG1 mutations in humans also suggest a more severe phenotype for ACTB mutations\(^33\),\(^35\) (phenotypes reviewed in the Supplementary Note and Supplementary Table 5). In contrast,
our findings suggest that both isoforms have overlapping but nonredundant functions during human development, as illustrated by the indistinguishable clinical presentations of individuals with Baraitser-Winter syndrome carrying mutations in ACTB and ACTG1 (Table 3 and Supplementary Table 6).

We examined protein levels and morphology in lymphoblastoid cell lines from six affected individuals. No differences were seen in β- and γ-actin protein levels between two representative cell lines derived from individuals carrying either the ACTB or ACTG1 recurrent mutation (encoding p.Arg196His and p.Ser155Phe, respectively) and controls (Fig. 2), similar to what was described for a previously reported ACTB mutation24. Comparable results were obtained with the four other cell lines with mutated actin genes (Supplementary Fig. 3). The two representative mutant cell lines both had greatly increased F-actin content and multiple, anomalous F-actin–rich, filopodia-like protrusions compared to control cells (Fig. 3a), resulting in increased cell perimeter (Fig. 3b). We probed the stability of F-actin using latrunculin A, which binds actin monomers, thereby preventing their incorporation into growing filaments36. Both representative mutant cell lines showed altered sensitivity to latrunculin A, albeit with different outcomes. We observed increased resistance to latrunculin A in mutant cells with the β-actin p.Arg196His alteration relative to controls, which is consistent with the effect of the previously reported p.Arg183Trp alteration34, whereas mutant cells with the γ-actin p.Ser155Phe alteration showed increased sensitivity to treatment (Fig. 3c). Immunofluorescent staining of F-actin in lymphoblastoid cell lines from the four other affected individuals showed patterns of cytoskeletal changes and abnormal accumulation of F-actin that were reproducible between independent cell lines carrying the same alteration (p.Arg196His in LR04-173 and LR09-079 and p.Ser155Phe in LP98-096 and LR04-298; Fig. 3 and Supplementary Fig. 4). Consistent with our initial analysis of mutant cell lines harboring the p.Arg196His or p.Ser155Phe alteration, all examined cell lines with the same mutation presented distinct patterns of abnormal F-actin organization relative to cell lines with other mutations, suggesting that morphologies are mutation specific.

Taken together, our data indicate that mutations associated with Baraitser-Winter syndrome result in increased F-actin content and altered F-actin dynamics in response to latrunculin A exposure. These effects are likely to have an impact on cell morphology, motility and other actin-related functions.

Several observations support a dominant-negative or gain-of-function mechanism for the disease-causing mutations. First, none of our subjects had deletions or protein-truncating mutations, which are an indication of haploinsufficiency. Furthermore, 11 of 18 mutations (61%) disrupted the same two amino acids (Arg196 in ACTB and Ser155 in ACTG1). Although these could be hypermutable sites,

| Sample ID | Chr. | Position (hg19) | Ref./alt. alleles | Gene | cDNA change | Amino-acid change | GERP score | Grantham score | CpG site | Inheritance | Other exomes |
|-----------|------|----------------|------------------|------|-------------|-----------------|------------|----------------|----------|-------------|-------------|
| LP98-085  | 7    | 5569255        | T/C              | ACTB | c.34A>G     | p.Asn12Asp      | 4.45       | 23             | –        | De novo     | 0/24        |
| LP90-050  | 7    | 5568962        | G/C              | ACTB | c.193C>G    | p.Leu65Val      | 4.33       | 32             | –        | De novo     | 0/244       |
| 61456     | 7    | 5568128        | G/A              | ACTB | c.586C>T    | p.Arg196Cys     | 0.50       | 180            | +        | N/A         | 0/214       |
| 58248a    | 7    | 5568127        | C/T              | ACTB | c.587G>A    | p.Arg196His     | 4.22       | 29             | +        | De novo     | 0/212       |
| 59169     | 7    | 5568127        | C/T              | ACTB | c.587G>A    | p.Arg196His     | 4.22       | 29             | +        | De novo     | 0/212       |
| LR04-173  | 7    | 5568127        | C/T              | ACTB | c.587G>A    | p.Arg196His     | 4.22       | 29             | +        | N/A         | 0/212       |
| LR09-079  | 7    | 5568127        | C/T              | ACTB | c.587G>A    | p.Arg196His     | 4.22       | 29             | +        | N/A         | 0/212       |
| LR06-298  | 7    | 5568127        | C/T              | ACTB | c.587G>A    | p.Arg196His     | 4.22       | 29             | +        | N/A         | 0/212       |
| 11-11287b | 7    | 5568127        | C/T              | ACTB | c.587G>A    | p.Arg196His     | 4.22       | 29             | +        | N/A         | 0/212       |
| 11-10221  | 7    | 5568127        | C/T              | ACTB | c.587G>A    | p.Arg196His     | 4.22       | 29             | +        | N/A         | 0/212       |
| 61488     | 17   | 79478933       | G/A              | ACTG1| c.359C>T    | p.Thr120Ile     | 4.16       | 89             | –        | De novo     | 0/244       |
| LR04-241  | 17   | 79478612       | GAA             | ACTG1| c.404C>T    | p.Ala135Val     | 2.95       | 64             | –        | De novo     | 0/192       |
| LR04-298  | 17   | 79478552       | G/A              | ACTG1| c.464C>T    | p.Ser155Phe     | 4.25       | 155            | –        | De novo     | 0/224       |
| LP98-096  | 17   | 79478552       | G/A              | ACTG1| c.464C>T    | p.Ser155Phe     | 4.25       | 155            | –        | De novo     | 0/224       |
| 11-10857  | 7    | 79478552       | G/A              | ACTG1| c.464C>T    | p.Ser155Phe     | 4.25       | 155            | –        | N/A         | 0/242       |
| 58431a    | 7    | 79478408       | G/T              | ACTG1| c.608C>T    | p.Thr203Ile     | 3.11       | 78             | –        | De novo     | 0/203       |
| LR03-033  | 7    | 79478256       | G/A              | ACTG1| c.760C>T    | p.Arg254Trp     | 0.08       | 101            | +        | De novo     | 0/195       |
| LP92-083a | 7    | 79478256       | G/A              | ACTG1| c.766C>T    | p.Arg256Trp     | 1.60       | 101            | +        | De novo     | 0/184       |

Chr., chromosome; ref., reference; alt., alternative; GERP, genomic evolutionary rate profiling; N/A, parents not available.

aSubjects analyzed by whole-exome sequencing. bSubject 11-11287 was originally diagnosed as having Fyns-Athimos syndrome20 (patient 1 in the original report; see Supplementary Note for phenotype analysis). cVariant reported in dbSNP (rs11549190). dPresence of the mutations in 244 control exomes sequenced at the University of Washington (see Online Methods for details), shown as number of samples carrying the variant over the total number of samples with bases covered by ≥2×.

Table 3 Clinical overview of individuals with Baraitser-Winter syndrome

|                      | Individuals with ACTB mutations (n = 10) | Individuals with ACTG1 mutations (n = 8) |
|----------------------|----------------------------------------|----------------------------------------|
| Growth               | Total%| Percentage | Total%| Percentage |
| Short stature        | 6/10 | 60         | 3/7   | 42.9       |
| Microcephaly, postnatal | 6/9 | 66.7       | 4/7   | 57.1       |
| Neurological         |       |            |       |            |
| Intellectual disability | 9/9 | 100        | 5/5   | 100        |
| Hearing loss         | 4/8  | 50         | 5/6   | 83.3       |
| Seizures             | 9/9  | 100        | 7/8   | 87.5       |
| Facial dysmorphism   |       |            |       |            |
| Trigonocephaly       | 8/10 | 80         | 7/7   | 100        |
| Hypertelorism        | 10/10| 100        | 7/8   | 87.5       |
| High-arched eyebrows | 10/10| 100        | 7/7   | 100        |
| Ptosis, congenital   | 10/10| 100        | 8/8   | 100        |
| Eyes                 |       |            |       |            |
| Coloboma (iris or retina) | 6/10 | 60         | 5/7   | 71.4       |
| Lissencephaly type   |       |            |       |            |
| Pachygryia- or pachygria-band | a-p | 8/8 | 100 | 7/7 | 100 |

a-p, anterior-to-posterior gradient of lissencephaly.

The number of affected individuals with the clinical feature over the total number of affected individuals for whom clinical data were available. See Supplementary Table 6 for a detailed clinical overview for each affected individual.

Fig. 3c) result-
mutation clustering suggests a gain-of-function effect, as observed in Noonan\(^2\), fibroblast growth factor receptor (FGFR)-related craniosynostosis\(^3\) and Proteus\(^4\) syndromes. Moreover, individuals with complete deletion or duplication of \(ACTB\) do not have Baraitser-Winter syndrome. To further analyze this, we searched the databases of two large clinical laboratories performing chromosome microarrays (Signature Genomic Laboratories and the Department of Human Genetics, Radboud University Nijmegen Medical Centre) and ascertained data from four children with \(ACTB\) or \(ACTG1\) alterations—two with a deletion and one with a duplication of \(7\)q22 that contains \(ACTB\) and one with a deletion of \(17\)q25.3 that contains \(ACTG1\). All four children lacked the major and most severe features of Baraitser-Winter syndrome, which are congenital ptosis and high-arched eyebrows, neuronal migration malformation and colobomata (Supplementary Fig. 5 and Supplementary Note). Finally, unchanged \(\beta\)- and \(\gamma\)-actin protein levels coupled with increased \(F\)-actin content observed in the lymphoblastoid cell lines derived from affected individuals also argue against haploinsufficiency.

In conclusion, our results show that Baraitser-Winter syndrome is a clinically well-defined disorder caused by mutations in the \(ACTB\) and \(ACTG1\) genes encoding cytoplasmic actin and provide additional evidence that actin has a central role in the pathogenesis of lissencephaly\(^{40,41}\). Our data also show that, even in a scenario of limited genetic heterogeneity, whole-exome sequencing of a small number of trios combined with validation in additional affected individuals is a powerful approach to identify mutations underlying well-defined sporadic disorders, especially those with low reproductive fitness. Because mutations in either \(ACTB\) or \(ACTG1\) result in the same phenotype, we hypothesize that mutations observed in Baraitser-Winter syndrome affect developmental functions shared by cytoplasmic \(\beta\)- and \(\gamma\)-actin. Functional comparison of mutations identified in Baraitser-Winter syndrome with those found in other actinopathies should help elucidate both the distinct and overlapping functions of cytoplasmic \(\beta\)- and \(\gamma\)-actin isoforms. We conclude that Baraitser-Winter syndrome comprises the severe end of the predominant phenotype spectrum associated with mutation of both \(ACTB\) and \(ACTG1\), a spectrum that extends from Baraitser-Winter syndrome to nonsyndromic hearing loss.

URLs. SeattleSeq Annotation 131, http://snp.gs.washington.edu/SeattleSeqAnnotation131/; Primer3, http://frodo.wi.mit.edu/primer3/; NIEHS Exome Project, http://evs.gs.washington.edu/niehsExome/.
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Written, informed consent was obtained from all subjects before enrollment in the study. Experiments on human subjects were approved by institutional review boards at all participating institutions. The study included 18 unrelated, affected individuals. The diagnosis of Baraitser-Winter syndrome was based on previously reported clinical features and review of MRI brain scans, when available. Genomic DNA was extracted from whole blood or saliva using standard methods. When parental DNA was available, paternity-maternity testing was performed by genotyping a panel of polymorphic short tandem repeats.

Exome sequencing. For trio 1, whole-blood genomic DNA from the affected proband and his parents (5 μg each) was sent to the University of Washington Genome Sciences Genomic Resource Center for exome capture and sequencing. Whole-exome sequence capture was performed using the SeqCap EZ Exome Library v2.0 liquid-phase sequence capture kit (Roche). Libraries were then sequenced on an Illumina Genome Analyzer Ix according to the manufacturer's recommendations for paired-end 76-bp reads. On average, we generated 81 million total reads (68 million mapped reads) and 5.2 Gb of mappable sequence per individual. We aligned reads to the human reference genome (hg19) with the Burrows-Wheeler Aligner (BWA) and retained only duplicate-paired reads. The Genome Analysis Toolkit (GATK) was used for base quality score recalibration and indel realignment, as well as for single-nucleotide variant and indel discovery and genotyping using standard hard filtering parameters. Variants with quality scores of <30, allele balance of >0.75, sequencing depth of >4, quality/density ratio of <0.5, length of homopolymer run of >5.0 and strand bias of >0.10 were flagged and excluded from subsequent analyses. Coverage was assessed with the GATK Depth of Coverage tool by ignoring reads with mapping quality of <20 and bases with base quality of <30. In total, 92% of the primary target was covered 24 times in all three individuals.

For trios 2 and 3, massively parallel sequencing of genomic DNA from two affected individuals—parent trios was performed at the Radboud University Nijmegen Medical Center by using the ABI SOLiD 4 platform (Life Technologies). Enrichment of exonic sequences was achieved by using the human SureSelect 50Mb set (Agilent), which targets ~21,000 human genes. On average, we obtained >120 million mappable sequencing reads and 4.6 Gb of mappable sequence data per individual after multiplex sequencing, using four exomes per sequencing slide. Color space reads were mapped to the hg19 reference genome with SOLiD Bioscope software version 1.3, which uses an iterative mapping approach. On average, 81% of bases originated from the targeted exome, resulting in a mean coverage of 64-fold (median of 66-fold). In total, 88% of the targeted exons were covered ≥10 times. Single-nucleotide variants were subsequently called by the DiBayes algorithm using high-stringency calling settings, and small insertions and deletions were detected using the SOLiD Small Indel Tool.

Discovery of de novo candidate variants. For trio 1, the 22,591 variants identified in the affected individual were annotated with SeattleSeq SNP annotation (see URLs). We focused on protein-altering variants (missense, nonsense and splice-site variants and coding indels) absent from dbSNP (build 132), 1000 Genomes Project data and 101 other exomes. From this list of rare variants, we identified potential de novo events using the following parameters: presence of ≥2 variant reads in the proband, sequencing depth at the variant position of ≥4 in both affected individuals and parental allelic balance of >0.90. Evolutionary conservation at the nucleotide level and impact of amino acid substitutions were assessed using GERP and Grantham matrix scores, respectively. Candidate de novo events were then inspected using Integrative Genomics Viewer (IGV) and validated in all three individuals by standard PCR reactions using custom primers designed with Primer3 and Sanger sequencing.

We identified a total of 28,497 and 29,685 variants in the probands of trios 2 and 3, respectively. Candidate de novo events were then verified using conventional Sanger sequencing.

Control exomes. For trio 1, variants were compared against 101 other exomes sequenced at the University of Washington, including 88 exomes from the NIEHS SNPs project (see URLs) and 13 exomes sequenced in the same batch as the trio and derived from healthy individuals and individuals with unrelated disorders. For trios 2 and 3, we used 332 other exomes sequenced at the Radboud University Nijmegen Medical Center to remove systematic artifacts and low-frequency variants.

We also analyzed the coding exons (±2 bp) of ACTB and ACTG1 in exome sequence data from 244 unrelated, healthy individuals of European descent who were parents of children with sporadic autism. These samples were sequenced at the University of Washington Genome Sciences using SeqCap EZ Exome Library v2.0 (Roche) and the Illumina sequencing platform. For all control samples combined, 89% of the bases of ACTB and ACTG1 were covered by ≥2×. The average per-base coverage was 23× and 31× for ACTB and ACTG1, respectively. The first coding exon of ACTB had a low read depth, with an average coverage of 4×; all other exons had sufficient coverage to call variants in the majority of samples. Variants were filtered to ≥8× coverage and consensus or variant quality of 30 using SAMtools, as previously described.

Identification of CNVs in control individuals. Genomic DNA samples from 2,349 control individuals (including 1,953 of European ancestry) was genotyped using the InfiniumII HumanHap610-Quad BeadChip array (Illumina) at the Center for Applied Genomics at the Children's Hospital of Philadelphia. Control subjects were healthy children ranging in age from 0–18 years who were primarily recruited from the Philadelphia region through the hospital's Health Care Network. CNVs were identified using the PennCNV algorithm and further filtered using a threshold of ten consecutive single-nucleotide variants, a length of 30 kb and a PennCNV confidence score of 10.

Sanger sequencing. We amplified the coding and flanking intronic regions of the ACTG1 (NM_001614) and ACTB (NM_000101) genes in genomic DNA from 18 probands. PCR products were sequenced at the University of Washington High-Throughput Genomics Unit or the Radboud University Nijmegen Medical Centre. All mutations were confirmed by re-amplification of the fragment and resequencing of the proband and his or her available relatives. Descriptions of primer sequences and PCR conditions are available on request.

SDS-PAGE, Coomassie staining and protein blots. Epstein-Barr virus-immortalized lymphoblastoid cell lines were established from peripheral blood samples of six affected individuals (LR04-173, LP98-096, LR09-079, LP98-085, LR04-298 and LR06-241) and two control individuals (09.1359 and 07.0841) using standard procedures. Cells were collected from suspension culture by centrifugation at 1,000g for 10 min. Cell pellets were rinsed once with PBS and lysed in M-PER lysis buffer (Pierce) supplemented with protease and phosphatase inhibitor cocktail (Sigma). The protein concentration of the lysates cleared of insoluble cell debris was determined using 660 nm Protein Assay reagent (Pierce), and more concentrated samples were diluted to the concentration of the least concentrated sample with lysis buffer. For SDS-PAGE, equal volumes of concentration-adjusted lysates were mixed with LDS electrophoresis loading buffer (Life Technologies) and reduced and denatured for 10 min at 70 °C. For one-dimension electrophoresis, equal volumes of reduced, denatured samples were separated on two 4–12% polyacrylamide gels (Life Technologies). One of the gels was processed for Coomassie staining (Bio-Rad), according to the manufacturer’s recommendations. The other gel was transferred onto 0.2-mm nitrocellulose membrane (Pall) and processed for protein blotting. Primary antibodies for protein blotting were used at the following dilutions: mouse antibody to ACTB (clone AC-15, Sigma), 1:32,000; mouse antibody to ACTG1 (clone 2-2.1.14.17, Sigma), 1:16,000; and goat antibody to GAPDH (sc-31915, Santa Cruz Biotechnology), 1:1,000. Appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad) were used at 1:40,000 dilutions. Proteins were detected by incubation with SuperSignal Pico (Pierce) chemiluminescent substrate, according to the manufacturer’s protocol.

Immunofluorescent staining of actin in cultured cells. Lymphoblastoid cells grown in suspension were placed on glass cover slips that had been coated
for 2 h with fibronectin (Millipore; 100 µg/ml) and laminin (Invitrogen; 25 µg/ml). After cells attached, experimental cultures were exposed to latrunoculin A (Cayman Chemical Company; 0.6 µM) for 2 h. Immediately after treatment, cells were fixed with 0.25% glutaraldehyde and permeabilized with 0.1% Triton X-100. Primary mouse antibody to α-tubulin (Sigma; 1:5,000) was applied overnight at 4 °C. Secondary Alexa Fluor–conjugated antibody (Life Technologies; 1:1,000) along with Alexa Fluor–conjugated phalloidin to visualize F-actin (Life Technologies; 1:100) was applied for 1 h at room temperature. Cover slips were mounted in ProLong Antifade medium (Life Technologies) and visualized with the 100× oil objective on an inverted microscope (Zeiss) fitted with a spinning-disc confocal scanner (PerkinElmer). All imaging analysis was performed using ImageJ software, where confocal stacks were projected into a single plane (Z-project, maximal intensity) and images were thresholded. The perimeter was measured as object perimeter, and fluorescence intensity was measured as a mean gray value.

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